APPENDIX D11

GAS GENERATION INFORMATION

Sandia National Laboratories

Albuquerque, New Mexico 87185-

date: January 26, 1996

to: Martin S. Tierney (6741)

from: Yifeng Wang & Larry Brush (Org. 6748)

subject: Estimates of Gas-Generation Parameters for The Long-Term WIPP Performance Assessment

Introduction

Steel corrosion and organic-material biodegradation have been identified as major gas-generation processes in the WIPP repository (Brush, 1995). Gas production will affect room closure and chemistry (Butcher, 1990; Brush, 1990). This memorandum provides the current estimates of gas-generation parameters for the long-term WIPP performance assessment. The parameters provided here include the rates of gas generation under inundated and humid conditions, the stoichiometric factors of gas generation reactions, and the probability of the occurrence of organic material biodegradation (Table 1). To satisfy the quality assurance (QA) requirement (QAP 9-5), we summarize all hand calculations for estimating these parameters in Appendices I and II.

Biodegradation of Organic Materials

Cellulosics, plastics, and rubbers have been identified as the major organic materials to be emplaced in the WIPP repository (DOE/CAO, 1996) and could be degraded by microbes in 10,000 years. Cellulosics has been demonstrated experimentally to be the most biodegradable among these materials (Francis et al., 1995). The occurrence of significant microbial gas generation in the repository will depend on: (1) whether microbes capable of consuming the emplaced organic materials will be present and active; (2) whether sufficient electron acceptors will be present and available; (3) whether enough nutrients will be present and available. Considering uncertainties in evaluation of these factors and also in order to bracket all possible effect of gas generation on the WIPP performance assessment, we assign a 50% probability to the occurrence of significant microbial gas generation.

- *Microbial Reactions*

Microorganism will consume cellulosics mainly via the following reaction pathways in the repository (Brush, 1995):

$$C_6H_{10}O_5 + 4.8\ H^+ + 4.8\ NO_3^- \rightarrow 7.4\ H_2O + 6\ CO_2 + 2.4\ N_2 \quad (1)$$

$$C_6H_{10}O_5 + 6\ H^+ + 3\ SO_4^{2-} \rightarrow 5\ H_2O + 6\ CO_2 + 3\ H_2S \quad (2)$$

$$C_6H_{10}O_5 \rightarrow 3\ CH_4 + 3\ CO_2. \quad (3)$$

We assume that Reactions 1 to 3 will proceed sequentially according to the energy yield of each reaction. Here we ignore the reaction pathways of aerobic respiration, Mn(IV) and Fe(III) dissimilatory reduction, since the quantities of O_2, Mn(IV) and Fe(III) initially present in the repository will be negligible relative to the other electron acceptors. In Reactions 1 to 3, biomass accumulation is also not taken into account. This is because significant biomass accumulation seems unlikely in the WIPP repository and the accumulated biomass, if any, will be recycled by microbes after all biodegradable cellulosics is consumed.

In addition to Reaction (3), methanogenesis may proceed via:

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O. \quad (4)$$

However, this reaction will be ignored in our calculations, because (1) no experimental data are available to evaluate the rate of this reaction and (2) the net effect of this reaction is to reduce the total gas generation and the amount of CO_2 in the repository and, therefore, it is conservative to ignore this reaction in respect of repository pressurization and actinide solubility.

- *Rates of Cellulosics Biodegradation*

The rate of cellulosics biodegradtion was measured by incubating representative cellulose materials (filter paper, paper towels, and tissue) in WIPP brine with microbes enriched from various WIPP environments (Francis & Gillow, 1994; Francis et al., 1995). The incubation experiments were conducted under various conditions: aerobic or anaerobic, inundated or humid, with or without bentonite, amended or unamended with nutrients or NO_3^-. Because the repository is expected to become anoxic shortly after waste emplacement and also because bentonite will not be added as a backfill according to the current waste emplacement plan, we think that the experimental data from anaerobic incubation without bentonite present are most relevant to expected WIPP conditions. Considering that the current experimental data are mostly for denitrification (Reaction 1), but not sulfate reduction (Reaction 2) and methanogenesis (Reaction 3) (Francis & Gillow, 1994; Francis et al., 1995), we assume that the ranges of the rates of cellulosics biodegradation via sulfate reduction and methanogenesis are equal to those observed for denitrification.

We use CO_2 production data to estimate the rates of cellulosics biodegradation. There are two advantages of using CO_2 production data: (1) there are experimental data available on the CO_2 dissolution in WIPP brine (Telander & Westerman, 1995) and, therefore, it is easy to correct the CO_2 production data for gas dissolution (Appendix I); (2) since cellulosics biodegradation did not reach the stage of methanogenesis in the experiments, according to

Reactions 1 and 2, the consumption of one mole carbon of cellulosics will produce one mole of CO_2. This 1:1 relationship is independent of oxidation state of carbon in cellulosics. Therefore, it is rather straightforward to determine the amount of cellulosics biodegraded from the amount of CO_2 produced.

Experimental data show a strong dependence of CO_2 generation on the concentrations of nutrients and nitrate (Francis & Gillow, 1994; Francis et al., 1995). The maximum CO_2 generation was observed in nitrate-and-nutrient-amended samples. In those experiments, after a short lag phase, CO_2 first linearly increased with time and then approached some limiting value as its production rate diminished. If we assume that biodegradation is nitrate- or nutrient-limited, the experimental data can be explained by Michaelis-Menton kinetics (Chapelle, 1993). Michaelis-Menton kinetics, which describes the dependence of microbial reaction rate on substrate concentration, can be expressed by:

$$V = \frac{V_{max} S}{K_s + S} \qquad (5)$$

where V is the microbial reaction rate; V_{max} is the maximum value of the rate; S is the concentration of the limiting substrate; K_s is a constant. Equation (5) states that the microbial reaction rate becomes independent of the substrate concentration, if the latter is high enough, i.e. $S >> K_s$ and $V = V_{max}$. In this circumstance, the reaction product will accumulate linearly with time before the substrate is sufficiently depleted. In other words, in our cases, the linear part of CO_2 vs. time curve will give the estimate of the maximum rate of cellulosics biodegradation.

From the experimental data of Francis & Gillow (1994) and Francis et al. (1995), we estimate the maximum and minimum rates of cellulosics biodegradation under inundated conditions to be 0.3 and 0.01 mole C/kg/year, respectively (Appendix I). The maximum rate is estimated from the data obtained from both NO_3^-- and nutrients-amended experiments, whereas the minimum rate is derived from the data obtained from the inoculated-only experiments without any nutrient and NO_3^- amendment. Under humid conditions, experimental data show no clear correlation between CO_2 production and nutrient concentration. The best estimate of the maximum rate of cellulosics biodegradation under humid condition is 0.04 mole C/kg/year (Appendix I). The minimum of the humid biodegradation rate is set to 0, corresponding to the cases where microbes become inactive due to nutrient and water stress.

- *Biodegradation of Plastics and Rubbers*

The rates of plastics and rubber biodegradation under expected WIPP conditions were

measured by Francis et al.(1995). The experimental data show that plastics and rubbers are much less biodegradable than cellulosics, although the data themselves are not sufficient for us to constrain the long-term biodegradation rate for plastics and rubbers. There are two factor that may potentially increase the biodegradibility of those materials: long time scale and cometabolism. Over a time scale of 10,000 years, plastics and rubbers may change their chemical properties and therefore their biodegradibility. Cometabolism means that microbes degrade an organic compound but do not use it as a source of energy or of their constituent elements, all of which are derived from other substrates (Alexander, 1994). In the WIPP repository, plastics and rubbers, which are resistant to biodegradation, may still be cometabolized with cellulosics and other more biodegradable organic compounds. Because of these uncertainties, we recommend a 50% chance for the biodegradation of plastics and rubbers in the event of significant microbial gas generation. We further suggest lumping plastics and rubbers into cellulosics and applying the ranges of cellulosics biodegradation rate to plastics and rubbers. This treatment is conservative in respect of repository pressurization and actinide solubility. We propose to use the following equation to convert plastics and rubbers to the carbon-equivalent quantity of cellulosics (Appendix I):

total cellulosics (kg) = actual cellulosics (kg) + 1.7 plastics (kg) + rubbers (kg). (6)

Anoxic Steel Corrosion

According to current waste inventory estimates, a large amount of steels will be emplaced in the WIPP repository (DOE/CAO, 1996). Those steels will be capable of reacting with the repository brine to form H_2 gas. Both thermodynamic calculations and experimental observations indicate that the H_2 gas can be generated to pressures exceeding the lithostatic pressure at the WIPP horizon, if enough brine enters the repository (Brush, 1990; Telander & Westerman, 1993, 1995). Since the repository will become anoxic shortly after waste emplacement and sealing, we here focus only on anoxic steel corrosion.

- *Steel Corrosion in the Absence of CO_2 and H_2S*

In this case, steel corrosion will follow the reaction (Telander & Westerman, 1993, 1995):

$$Fe + 2\,H_2O \rightarrow Fe(OH)_2 + H_2. \quad (7)$$

In the Mg-rich WIPP brines (exemplified by Brine A), a significant fraction of Fe in the corrosion product is substituted by Mg. This substitution can substantially increase the stability of the corrosion product. Experimental observations indicate that steel corrosion can still proceed even at an 127 atm H_2 pressure (Telander & Westerman, 1995). Aside from this thermodynamic stability argument, the experimental observations indicate no

essential effect of Mg in the brine on the corrosion rate. As a matter of fact, the corrosion rates measured in Mg-rich Brine A are not significantly different from those measured in Mg-depleted Brine ERDA-6 (Telander & Westerman, 1995).

It was observed in the experiments that the steel corrosion rate decreased with time until some limiting rate was achieved (Telander & Westerman, 1995). Our long-term corrosion rate is estimated from the longest-term data available in a WIPP-relevant Brine A environment. The estimated inundated rate is 0.5 μm/year or 0.07 mole Fe /m^2/year (Appendix II). In addition, the corrosion rate is also found to increase with decreasing brine pH (Telander & Westerman, 1993, 1995). Without addition of CO_2 from microbial reactions, the pH in the repository is unlikely to go below its experimental value, which is about 10 (Telander & Westerman, 1993, 1995). Therefore, we recommend using 0.5 μm/year as the upper limit of inundated corrosion rate for the cases without microbial gas generation. On the other hand, the pH in the repository can be ~2 units higher than its experimental value due to the presence of $Ca(OH)_2$ as a cementious material in the waste, and thus, based on the scaling factor (= 0.01) given by Telander & Westerman (1995), the steel corrosion rate could be as low as 0.005 μm/year. In addition, the experimental work for Source Term Test Program (STTP) at Los Alamose National Laboratory indicates that salt crystallization on steel surface may possibly prevent the steel from corrosion. To include this possibility, we set the minimum inundated steel corrosion rate to 0.

The corrosion rate observed on specimens exposed to humid conditions is negligible, based on essentially non-existent presence of corrosion product and lack of apparent H_2 generation (Telander & Westerman, 1995). Therefore, we set the humid steel corrosion rate to 0.

- *Steel Corrosion in the Presence of CO_2 and H_2S*

In the event of significant microbial gas generation, steel corrosion can proceed via the following reactions in addition to Reaction (7) (Telander & Westerman, 1993, 1995):

$$Fe + CO_2 + H_2O \rightarrow FeCO_3 + H_2 \quad (8)$$
$$Fe + H_2S \rightarrow FeS + H_2. \quad (9)$$

One possible effect of CO_2 and H_2S on steel corrosion is that they may cause passivation of the steel. Steel passivation was observed in the experiments in which large quantities of CO_2 and H_2S were added to the reaction vessels. It usually took place after tens of days and was caused by the formation of a protective layer of $FeCO_3$ or FeS on steel surfaces (Telander & Westerman, 1995). However, we think that this passivation is unlikely to occur under the repository conditions. This is because the microbial production rate of CO_2 and H_2S is too slow and it will take an exceedingly long time period (relative to the experimental time scale) for these gases in the repository to reach their concentration levels required for

passivation under the experimental conditions. The conclusion of no steel passivation under the WIPP repository conditions is consistent with other studies (e.g., Ikeda et al., 1983; Schmitt, 1983). In fact, aside from the previously cited work of Telander & Westerman (1993), total passivation of steel by CO_2 and H_2S in low-temperature solutions has not been reported, though varying degrees of corrosion inhibition have been observed.

In the absence of passivation, the microbial generation of CO_2 and H_2S will increase steel corrosion rates in the repository either by lowering the repository pH or by initiating additional reaction pathways (Reactions 8 and 9) (Telander & Westerman, 1995). We take this effect into account by modifying the sampling range of steel corrosion rate. Obviously, Reactions 8 and 9 will be limited by microbial CO_2 and H_2S production, and therefore the upper limit of the reaction rate can be estimated from the maximum cellulosics biodegradation rate, which is 0.3 mole/kg cellulosics/year, equivalent to 6 μm/year of steel corrosion rate (Appendix II). Thus, in the event of significant microbial gas generation, the upper limit of steel corrosion rate is 6.5 μm/year, the sum of the maximum rates of Reactions 7 through 9. The corresponding lower limit will be kept the same as that estimated for the cases without CO_2 production, i.e. 0.0 μm/year. Under humid conditions, experimental results show a negligible effect of CO_2 and H_2S on steel corrosion (Telander & Westerman, 1995). We thus set the humid corrosion rate to 0.

Stoichiometric Factors in the Average-Stoichiometry Model

In the Average-Stoichiometry Model, which is currently implemented in BRAGFLO, microbial gas generation is represented by the overall reaction:

$$\frac{1}{6}C_6H_{10}O_5 + \textit{unknown} \rightarrow y\ \textit{gas} + \textit{unknown}$$

and H_2 production due to steel corrosion is described by:

$$\text{Fe} + \frac{4+2x}{3}\,\text{H}_2\text{O} \rightarrow \frac{4-x}{3}\,\text{H}_2 + x\,\text{Fe(OH)}_2 + \frac{1-x}{3}\,\text{Fe}_3\text{O}_4.$$

The stoichiometric factors x and y in Reaction 10 and 11 are estimated as follows.

- *Average-Stoichiometric FactorY in Microbial Reaction*

The stoichiometric factor y depends on the extent of the progress of each individual reaction pathway (Reactions 1 through 3). It can be estimated based on the inventory estimates of the transuranic waste to be emplaced in the Waste Isolation Pilot Plant (DOE/CAO, 1996; Drez, 1996).

First, we estimate the maximum quantities (in moles) of cellulosics and steels that will be potentially consumed in 10,000 years:

$$\min \left\{ \frac{6000 M_{cel}}{162},\ 10000 R_C' M_{cel} \right\} \tag{12}$$

$$M_{Fe}' = \min \left\{ \frac{1000 M_{Fe}}{56},\ 1410 R_{c,i} A \right\} \tag{13}$$

$$R_c' = \max \left\{ R_{m,i},\ R_{m,h} \right\} \tag{14}$$

where M_{cel} and M_{Fe} are the quantities (in kg) of cellulosics and steels initially present in the repository; $R_{c,i}$ is the inundated steel-corrosion rate (μm/year); $R_{m,i}$ and $R_{m,h}$ are the sampled rates of cellulosics biodegradation under inundated and humid conditions respectively (mole/kg/year). In Equation (13), we use the factor of 0.141 mole/μm/m^2 to convert steel-corrosion-rate unit from μm/year to mole/m^2/year (Telander and Westerman, 1995). Here, we assume that cellulosics biodegradation and steel corrosion both follow zero order reaction kinetics. Next, we calculate the average stoichiometric factor y by distributing M_{cel}' into individual biodegradation pathways. Consider two extreme cases, corresponding to the maximum and minimum values of y: (1) no reaction of microbially produced CO_2 and H_2S with steel and steel-corrosion products; (2) complete reaction of microbially produced CO_2 and H_2S with steel and steel-corrosion products.

If no CO_2 or H_2S is consumed by reactions with steel and steel-corrosion products, we would expect the maximum quantity of microbial gas production in the repository and therefore the maximum value for y. We assume that Reactions 1 to 3 will proceed sequentially. The maximum value of y can be estimated by averaging the gas-yields for all reaction pathways:

$$y_{max} = \frac{\frac{8.4 M_{NO3}}{4.8} + \frac{9 M_{SO4}}{3} + \left(M_{cel}' - \frac{6 M_{NO3}}{4.8} - \frac{6 M_{SO4}}{3} \right)}{M_{cel}'} \tag{15}$$

where M_{NO3} and M_{SO4} are the quantities of NO_3^- and SO_4^{2-} (in moles) initially present in the repository.

If CO_2 or H_2S reacts with steel and steel-corrosion products, we expect that a significant quantity or, perhaps, all of these microbially produced gases would be consumed, thus forming $FeCO_3$ and FeS. This would result in the minimum value of y. The total gas consumed by those reactions (G) is:

$$G = \min\left\{\frac{6M_{NO3}}{4.8} + \frac{9M_{SO4}}{3} + \frac{3}{6}\left(M'_{cel} - \frac{6M_{NO3}}{4.8} - \frac{6M_{SO4}}{3}\right),\ M'_{Fe}\right\} \tag{16}$$

The minimum value of y can then be estimated by:

$$y_{min} = \frac{\frac{8.4M_{NO3}}{4.8} + \frac{9M_{SO4}}{3} + \left(M'_{cel} - \frac{6M_{NO3}}{4.8} - \frac{6M_{SO4}}{3}\right) - G}{M'_{cel}} = y_{max} - \frac{G}{M'_{cel}}$$

For each BRAGFLO simulation, *y* will be uniformly sampled over $[y_{min}, y_{max}]$:

$$y = y_{min} + \beta(y_{max} - y_{min})$$

with $0 \le \beta \le 1.0$. The calculational scheme proposed here automatically correlates *y* with waste inventory estimates as well as with reaction rates.

The above calculational scheme does not take into account the SO_4^{2-} that will be brought into repository by brine inflow. Based the previous BRAGFLO simulations for undisturbed cases, the total volume of the brine entering the repository in 10000 years is unlikely to be larger than 2.2×10^7 liters, the value corresponding to the case with unrealistically low gas generation and therefore the worst repository flooding. With a typical SO_4^{2-} concentration of 200 mM in WIPP brines (Brush, 1990), we estimate that the amount of SO_4^{2-} brought into the repository by brine inflow would be less than 0.4×10^7 moles. This amount of SO_4^{2-} will increase the fraction of sulfate reduction pathway in total cellulosics biodegradation only by less than 1%. Therefore, neglecting the sulfate brought by brine inflow would introduce an error of no more than a few percents in y values.

• *Average-Stoichiometric FactorX in Steel Corrosion Reaction*

While magnetite (Fe_3O_4) has been observed to form on steel as a corrosion product in low-Mg anoxic brines at elevated temperatures (Telander & Westerman, 1995) and in oxic brine (Haberman & Frydrych, 1988), there is no evidence that it will form at WIPP repository temperatures. If Fe_3O_4 were to form, it would be expected that H_2 would be produced (on a molar basis) in excess of Fe consumed. But, the anoxic corrosion experiments did not show the production of H_2 in excess of Fe reacted. Therefore, we set the stoichiometric factor x to 1.0 in Reaction 11.

Table 1. Gas-Generation Parameters for the Long-Term WIPP Performance Assessment

Parameter	Estimated Value
Probability of occurrence of significant microbial gas generation	50%
Probability of occurrence of plastics and rubber biodegradation in the event of significant gas generation	
Rate of inundated cellulosics biodegradation	0.01 - 0.3 mole C/kg/year
Rate of humid cellulosics biodegradation	0.0 - 0.04 mole C/kg/year
Rate of inundated steel corrosion for the cases without microbial gas generation	0.0 - 0.5 μm/year[1]
Rate of humid steel corrosion for the cases without microbial gas generation	0.0 μm/year
Rate of inundated steel corrosion for the cases with microbial gas generation	0.0 - 6.5 μm/year
Rate of humid steel corrosion for the cases with microbial gas generation	0.0 μm/year
Stoichiometric factor x in Reaction 11	
Stoichiometric factor y in Reaction 10	calculated from Eqn. (18)
Factor β in Equation 18	0 - 1.0
NO_3^- initially present in the waste[2]	2.6×10^7 moles
SO_4^{2-} initial present in the waste[2]	6.6×10^6 moles

1. Multiplying 0.141 mole/μm/m^2 will convert the unit of steel corrosion rate from μm/year to mole/m^2/year (Telander & Westerman, 1993). 2. See Appendix I.6.

References

Alexander M. (1994) *Biodegradation and Bioremediation.* Academic Press, NY.

Brush L. H. (1990) *Test Plan for Laboratory and Modeling Studies of Repository and Radionuclide Chemistry for the Waste Isolation Pilot Plant.* SAND90-0266, Sandia national Laboratories, Albuquerque, NM.

Brush L. H. (1995) *Systems Priority Method - Iteration 2 Baseline Position Paper: Gas Generation in the Waste Isolation Pilot Plant.* Sandia National Laboratories, Albuquerque, NM.

Butcher B. M. (1990) *Preliminary Evaluation of Potential Engineered Modifications for the Waste Isolation Pilot Plant (WIPP).* SAND89-3095, Sandia National Laboratories, Albuquerque, NM.

Chapelle F. H. (1993) *Ground-Water Microbiology and Geochemistry.* John Wiley & Sons, NY.

DOE/CAO (1996) *Transuranic Waste Baseline Inventory Report.* (Rev. 2)

Drez P. (1996) Preliminary estimate for SNL/NM performance assessment calculations of nitrate, sulfate, and phosphate content in transuranic solidified wastes destine for disposal in WIPP. CTAC. (memo, 1/26/1996)

Francis A. J & Gillow J. B. (1994) *Effects of Microbial Processes on Gas Generation under Expected WIPP Repository Conditions: Progress Report through 1992.* SAND93-7036, Sandia National Laboratories, Albuquerque, NM.

Francis A. J., Gillow J. B. & Giles M. R. (1995) *Effects of Microbial Processes on Gas Generation under Expected WIPP Repository Conditions: Data Report for the Period 1993 - 1995.* (draft)

Haberman J. H. & Frydrych D. J. (1988) Corrosion studies of A216 Grade WCA steel in hydrothermal magnesium-containing brines. *Scientific Basis for Nuclear Waste Management XI, Materials Research Society Symposium Proceedings, Boston, MA, November 30 - December 3, 1987.* Eds. M. J. Apted and R. E. Westerman. Materials Research Society, Pittsburgh, PA. 112, 761-772.

Ikeda A., Ueda M. & Mukai S. (1983) CO_2 corrosion behavior and mechanism of carbon steel and alloy steel. *Corrosion '83, Anaheim, CA, April 18 - 22, 1983.* Paper no. 45. National Association of Corrosion Engineers, Huston, TX.

Molecke M. A. (1979) *Gas Generation from Transuranic Waste Degradation: Data Summary and Interpretation.* SAND79-1245, Sandia National Laboratories, Albuquerque, NM.

Schmitt G. (1983) CO_2 corrosion of steels - An attempt to range parameters and their effects. *Advances in CO_2 Corrosion in the Oil and Gas Industry, Proceedings of the*

Corrosion/83 Symposium, Anaheim, CA, April 18 - 19. Ed. R. H. Hausler. National Association of Corrosion Engineers, Huston, TX. 1, 1-2.

Telander M. R. & Westerman (1993) *Hydrogen Generation by Metal Corrosion in Simulated Waste Isolation Pilot Plant Environments: Progress Report for the Period November 1989 through December 1992.* SAND92-7347, Sandia National Laboratories, Albuquerque, NM.

Telander M. R. & Westerman (1995) *Hydrogen Generation by Metal Corrosion in Simulated Waste Isolation Pilot Plant Environments: Final Report.* (draft)

THIS PAGE LEFT BLANK INTENTIONALLY

Appendix I. Hand Calculations for Estimating Microbial Gas generation Parameters

Name of person performing the calculations: Yifeng Wang, Jan. 26, 1996 Yifeng Wang
Name of person reviewing the calculations: Larry Brush, Jan. 26, 1996 Larry Brush

I.1 Correction for CO_2 Dissolution in the Brine

Data and definition of variables:

TCO_2: Total CO_2 produced in an incubating experiment (moles)
n: Measured CO_2 in headspace (moles)
C_l: Dissolved CO_2 (moles/l)
V_l: Brine volume = 0.104 (l) (Gillow, per. comm.)
V_g: Headspace volume 0.046 (l) (Francis & Gillow, 1994)
P: Partial pressure of CO_2 (atm)
K: Partition coefficient of CO_2 between brine and gas phase = 0.01 (mole/l/atm) (Telander & Westerman, 1995)
R: Gas constant = 0.082 (l•atm/mole/K)
T: Temperature = 303.15 (K)

Assumption: Gaseous CO_2 approximately follows the idea gas law during these experiments.

Calculations:

$TCO_2 = V_l*C_l + n = K*P*V_l + n = K*V_l*n*R*T/V_g + n = (K*V_l*R*T/V_g + 1)*n =$
$(0.01*0.104*0.082*303.15/0.046 + 1)*n = 1.56*n$ (moles).

I.2 Estimate of the Maximum Inundated Cellulosics Biodegradation Rate

Data:

Source: Francis et al. 1995, p. 41, 148-149.
Experimental conditions: anaerobic inoculated, nutrients and nitrate amended

We only take the linear part of CO_2 vs. time curve:

time	CO_2
69 days	6.1 μmol/g of cel.
411 days	163 μmol/g of cel.

Calculations:

(1) Rate = (163 - 6.1)/(411 - 69) = 0.459 micro-moles/g/day = 0.168 mole/kg/year.
(2) Correcting it for dissolved CO_2 (see I.1), we finally have:
maximum rate = 0.168*1.56 = 0.3 mole/kg/year.

I.3 Estimate of the Minimum Inundated Cellulosics Biodegradation Rate

Data:
Source: Francis et al. 1995, p. 148-149.
Experimental conditions: anaerobic, inoculated only,

time	CO_2
0 days	2.1 µmol/g of cel.
1034 days	14.0 µmol/g of cel.

Calculations:
(1) Rate = (14.0 - 2.1)/(1034 - 0) = 0.0115 µmol/g/day = 0.004 mole/kg/year.
(2) Correcting it for dissolved CO_2 (Appendix I.1), we finally have:
minimum rate = 0.004*1.56 = 0.01 mole/kg/year.

I.4 Estimate of the Maximum Humid Cellulosics Biodegradation Rate

Data:
Source: Francis et al. 1995, p. 80.
Experimental conditions: anaerobic, inoculated only;
anaerobic, inoculated and amended

time	CO_2
6 days	(7.7 + 13.3)/2 = 10.5 µmol/g of cel.
415 days	(83.1 + 28.8)/2 = 56 µmol/g of cel.

Calculations:
Maximum rate = (56 - 10.5)/(415 - 6) = 0.11µmol/g/day = 0.04 mole/kg/year.

I.5 Convert Plastics and Rubbers to the Equivalent Quantity of Cellulosics

Data:

Source: Molecke (1979)

Celllulosics: $C_6H_{10}O_5$	M. W. = 162 g/mole
Polyethylene: $(-C_2H_4-)n$	M. W. = 28 g/mole
Polyvinychloride: $(-C_2H_3Cl-)n$	M. W. = 62 g/mole
Neoprene: $(-C_4H_5Cl-)n$	M. W. = 88 g/mole
Hypalon: $(-(C_7H_{13}Cl)_{12}-(CHSO_2Cl)_{17}-]n$	M. W. = 3488 g/mole

Assumption:

Plastics: 80% polyethylene, 20% polyvinychloride
Rubbers: 50% neoprene, 50% hypalon
Based on Molecke (1979).

Calculations:

The P kilograms of plastics and R kilograms of rubbers are equivalent to the Q kilograms of cellulosics, based on carbon equivalence:

$Q = (0.8*2*162/28/6 + 0.2*2*162/62/6)*P + (0.5*4*162/88/6 + 0.5*101*162/3488/6)*R = 1.7\,P + R$ (kilograms)

I.6 Moles of NO_3^- and SO_4^{2-} Initially Present in the Waste

NO_3^-: 1.62×10^6 kg (Drez, 1996) = $1000/62*1.62\times10^6 = 2.6\times10^7$ moles
SO_4^{2-}: 6.33×10^5 kg (Drez, 1996) = $1000/96*6.33\times10^5 = 6.6\times10^6$ moles

Appendix II. Hand Calculations for Estimating Steel Corrosion Parameters

Name of person performing the calculations: Yifeng Wang, Jan. 26, 1996 Yifeng Wang
Name of person reviewing the calculations: Larry Brush, Jan. 26, 1996 Larry Brus

II.1 Estimate of the Maximum Inundated Steel Corrosion Rate for the Cases without Microbial Gas Generation

Data:

(1) Anoxic corrosion rate obtained from the 12th to 24th month experimental data = 0.71 μm/year (Telander & Westerman, 1993, p. 6-14).

(2) Scaling factor for the long-term rate = 70% (Telander & Westerman, 1995, p. 6 - 19).

Calculation:

The maximum long-term steel corrosion rate = 0.71*70% = 0.5 μm/year.

II.2 Estimate the Maximum Inundated Steel Corrosion Rate for the Cases with Microbial Gas Generation

Data:

Total transuranic waste volume: 1.5×10^5 m^3 (DOE/CAO, 1996)
Drum volume: 0.208 m^3 (DOE/CAO, 1996)
Surface area of steel: 6 m^2/drum (Brush, 1995)
Maximum cellulosics biodegradation rate: 0.3 mole/kg/year (Appendix I.2)
Maximum inundated steel corrosion rate for the cases without microbial gas generation: 0.5 μm/year.
Total cellulosics (including plastics and rubbers): 2.1×10^7 kg (DOE/CAO, 1996; Appendix I.5)
NO_3^- initially present in the waste: 2.6×10^7 moles (Appendix I.6)
SO_4^{2-} initial present in the waste: 6.6×10^6 moles (Appendix I.6)

Assumption:

Reactions 8 and 9 will be limited by microbial CO_2 and H_2S production rate.

Calculations:

(1) Number of drums = $1.5 \times 10^5 / 0.208 = 7.2 \times 10^5$ drums.

(2) Total moles of C in cellulosics = $6*2.1 \times 10^7 * 1000/162 = 7.74 \times 10^8$ moles of C.

Molar fraction of cellulosics biodegraded via denitrification = $2.6 \times 10^7 / 7.74 \times 10^8 = 3\%$.

Molar fraction of cellulosics biodegraded via sulfate reduction = $6.6 \times 10^6 / 7.74 \times 10^8 = 1\%$.

(3) Maximum CO_2 and H_2S production rate for the whole repository = $(0.03 + 1.5*0.01 + 0.5*0.96)*0.3*2.1 \times 10^7 = 3.3 \times 10^6$ moles CO2/year.

(4) Total steel surface area = $6*7.2 \times 10^5 = 4.32 \times 10^6$ m^2.

(5) The maximum rate of steel corrosion via Reactions 8 and 9 = $3.3 \times 10^6 / 4.32 \times 10^6 =$ 0.8 mole Fe/m^2/year = 6 μm/year.

(6) The upper limit of inundated steel corrosion rate for the cases with microbial gas generation = 0.5 + 6 = 6.5 μm/year.

Distribution:

MS 1320 J. Nowak (Org. 6831)
MS 1320 R. V. Bynum (Org. 6831)
MS 1328 P. Vaughn (Org. 6749)
MS 1328 D. R. Anderson (Org. 6749)
MS 1328 M. S. Tierney (Org. 6741)
MS 1328 H. N. Jow (Org. 6741)
MS 1335 M. S. Y. Chu (Org. 6801)
MS 1341 J. T. Holmes (Org. 6748)
MS 1341 L. H. Brush (Org. 6748)
MS 1341 Y. Wang (Org. 6748)
MS 1330 SWCF (Org. 6352), WBS 1.1.09.1.1(R)

THIS PAGE LEFT BLANK INTENTIONALLY

Sandia National Laboratories

Albuquerque, New Mexico 87185-

date: February 29, 1996

to: Palmer Vaughn (Org. 6749)

from: Yifeng Wang & Larry Brush (Org. 6748)

subject: An Adjustment for Using Steel Corrosion Rates in BRAGFLO to Reflect Repository Chemical Condition Changes due to Adding MgO as a Backfill

In order to control the repository chemistry, a sufficient amount of MgO will be added to the repository as a backfill. Through chemical reaction, this backfill will practically remove all CO_2 generated by microbial reactions and thus prevent any possibility of CO_2 accumulation in the repository. Therefore, the previously-suggested enhancement of steel corrosion by CO_2 (Wang & Brush, 1996) will be no longer possible. In our previous memo (Wang & Brush, 1996), two set of inundated steel corrosion rates were provided: one is 0.0 to 0.5 μm/year for the cases without CO_2 present and another is 0.0 to 6.5 μm/year for the cases with CO_2 present. *Considering the chemical condition changes due to adding MgO as a backfill, we suggest using the rate of 0 to 0.5 μm/year for all BRAGFLO simulations.*

Distribution:

R. E. Westerman (PNL)
MS 1320 J. Nowak (Org. 6831)
MS 1320 R. V. Bynum (Org. 6831)
MS 1328 P. Vaughn (Org. 6749)
MS 1328 D. R. Anderson (Org. 6749)
MS 1328 M. S. Tierney (Org. 6741)
MS 1328 H. N. Jow (Org. 6741)
MS 1335 M. S. Y. Chu (Org. 6801)
MS 1341 J. T. Holmes (Org. 6748)
MS 1341 L. H. Brush (Org. 6748)
MS 1341 Y. Wang (Org. 6748)
MS 1330 SWCF (Org. 6352), WBS 1.1.09.1.1(RC)

THIS PAGE LEFT BLANK INTENTIONALLY

CONTRACTOR REPORT

SAND93—7036
Unlimited Release
UC—721

Effects of Microbial Processes on Gas Generation Under Expected Waste Isolation Pilot Plant Repository Conditions

Progress Report Through 1992

A. J. Francis and J. B. Gillow
Brookhaven National Laboratory
Upton, NY 11973

Prepared by Sandia National Laboratories Albuquerque, New Mexico 87185 and Livermore, California 94550 for the United States Department of Energy under Contract DE-AC04-94AL85000

Printed April 1994

Issued by Sandia National Laboratories, operated for the United States Department of Energy by Sandia Corporation.

Available to DOE and DOE contractors from
Office of Scientific and Technical Information
PO Box 62
Oak Ridge, TN 37831

Prices available from (615) 576-8401, FTS 626-8401

Available to the public from
National Technical Information Service
US Department of Commerce
5285 Port Royal Rd
Springfield, VA 22161

NTIS price codes
Printed copy: A07
Microfiche copy: A01

SAND93-7036
Unlimited Release
Printed April 1994

Distribution
Category UC-721

EFFECTS OF MICROBIAL PROCESSES ON GAS GENERATION UNDER EXPECTED WASTE ISOLATION PILOT PLANT REPOSITORY CONDITIONS

Progress Report Through 1992

A. J. Francis and J. B. Gillow
Brookhaven National Laboratory
Upton, NY 11973

ABSTRACT

Microbial processes involved in gas generation from degradation of the organic constituents of transuranic waste under conditions expected at the Waste Isolation Pilot Plant (WIPP) repository are being investigated at Brookhaven National Laboratory. These laboratory studies are part of the Sandia National Laboratories - WIPP Gas Generation Program. Gas generation due to microbial degradation of representative cellulosic waste was investigated in short-term (< 6 months) and long-term (> 6 months) experiments by incubating representative paper (filter paper, paper towels, and tissue) in WIPP brine under initially aerobic (air) and anaerobic (nitrogen) conditions. Samples from the WIPP surficial environment and underground workings harbor gas-producing halophilic microorganisms, the activities of which were studied in short-term experiments. The microorganisms metabolized a variety of organic compounds including cellulose under aerobic, anaerobic, and denitrifying conditions. In long-term experiments, the effects of added nutrients

---

Performed under Contract No. 67-8602 for Sandia National Laboratories, Waste Isolation Pilot Plant Gas Generation Program.

(trace amounts of ammonium nitrate, phosphate, and yeast extract), nutrients plus excess nitrate, and no nutrients on gas production from cellulose degradation were investigated. Results to date (up to 200 days of incubation) show that: (i) gas production was not detected in abiotic control samples; (ii) cellulose incubated without nutrients showed limited but sustained gas production; (iii) the addition of nutrients enhanced the biodegradation of cellulose as evidenced by an increase in the production of total gas, carbon dioxide, and nitrous oxide; (iv) in the presence of excess nitrate, gas production was the highest and nitrous oxide accumulated to varying amounts; (v) the addition of bentonite increased the background carbon-dioxide concentration and stimulated microbial activity specifically in aerobic samples; and (vi) in addition to total gas and carbon dioxide production, cellulose degradation in nutrient-amended samples was evidenced by the gradual bleaching of brown paper towel, the formation of gas bubbles, the formation of paper pulp, and the appearance of a red color at the bottom of the sample bottles, indicating the growth of halophilic microorganisms. Estimates of the total gas production on the basis of initial results ranged from 0.001 to 0.039 mL g^{-1} cellulose day^{-1}.

ACKNOWLEDGMENTS

The authors acknowledge the encouragement and programmatic guidance provided by Drs. L. H. Brush and M. A. Molecke, Sandia National Laboratories; the technical assistance of J. Ruggieri and S. Melloy; P. Carr and V. Gutierrez for their assistance with the Quality Assurance aspects of the project; A. Woodhead for editorial assistance; and C. Messana for preparing the final report.

CONTENTS

CONTENTS (Continued)

1.0 INTRODUCTION

Transuranic (TRU) wastes contain alpha-emitting transuranium nuclides with half-lives greater than twenty years and concentrations greater than 100 nCi per gram. TRU wastes are generated from nuclear-weapons production and other related nuclear-processing procedures. The wastes include adsorbed liquids, sludges, organics, and cemented materials containing the following radionuclides: ^{232}Th, ^{233}U, ^{235}U, ^{238}U, ^{237}Np, ^{238}Pu, ^{239}Pu, ^{240}Pu, ^{241}Pu, ^{242}Pu, ^{241}Am, ^{244}Cm, ^{252}Cf, and a variety of metals. Typically, TRU waste is classified as either contact-handled (CH), which does not require shielding, or remote-handled (RH), which requires shielding because of the hazard of gamma-radiation exposure. The Waste Isolation Pilot Plant (WIPP) is a mined, geologic repository developed to demonstrate that radioactive transuranic wastes generated in defense-related activities can be safely and permanently disposed of underground. The WIPP is a U.S. Department of Energy facility located in southeastern New Mexico, about 2150 ft (656 m) below the surface, in a bedded salt, evaporite Permian formation. A major long-term concern is the potential for gas generation from the corrosion of Fe and Fe-based alloys, microbial degradation of cellulosic, plastic, and rubber materials, and radiolysis of brine and waste material by alpha-emitting radionuclides (Molecke, 1979; Brush, 1990). Gas generation can cause pressurization and the formation of fractures which could allow the radionuclides to migrate away from the disposal site.

Anoxic corrosion and microbiological activity are the two most important processes that may generate appreciable amounts of gas. The current estimate of gas production due to anoxic corrosion is 900 moles per drum of waste (Brush, 1991). Caldwell et al. (1979) reported that microbial gas production due to biodegradation of TRU waste could be significant. Recently, Lappin et al. (1989) estimated such production rates at 1 mole of gas per drum of waste per year for 600 years. Brush (1991) and Brush et al., (1990) proposed a minimum and maximum range at 0 to 5 moles per drum per year, as did the earlier estimates by Molecke (1979).

Laboratory studies are under way to determine the rate and extent of gas production due to radiolysis, corrosion, and microbial activity, to support the Sandia National Laboratories-WIPP Gas Generation Program efforts to assess the long-term performance of the WIPP repository. This report summarizes the progress and status of the WIPP-funded work at BNL and presents the microbiological data obtained from initiation through 1992. Studies of the effects of microbial processes have been underway at Brookhaven National Laboratory since 1991, funded under Sandia National Laboratories contract no. 67-8602.

Brookhaven National Laboratory has developed a Quality Assurance Program that complies with DOE Order 5700.6C. For EM projects the Laboratory interprets the requirements of 5700.6C in accordance with the applicable guidance provided in the EM Quality Assurance Requirements Document (QARD). This will ensure that the data generated will be valid, accurate, repeatable, and protected, and will withstand critical peer and other reviews.

1.1 Background

The WIPP waste repository is located 2150 ft (656 m) below ground surface, with 56 rooms planned or under construction in a bedded salt formation. About 6,800 drums of waste in 55-gallon (208-L) containers will be placed in each room of 3,640-m^3 capacity. Each drum will contain, on average, about 10 kg of cellulosic waste (approx. 70,000 kg of cellulosic per room), 70% of which is paper (Brush, 1990). The rest of the potentially biodegradable portion of the waste consists of plastic and rubber, and other organic compounds. Wastes consisting of inorganic process sludges from secondary waste treatment, containing a total of ~3 million moles of nitrate and a much smaller amount of phosphate, will also be emplaced in the WIPP (Brush, 1990; Brush et al., 1991).

Microorganisms can enter the WIPP from several sources, including: (i) association with the TRU waste; (ii) the surface environment via the mine ventilation systems and human intrusion; and (iii) resident populations in the salt crystals and brine formations. Alpha

radiation from TRU waste is not expected to have significant effects on microbial activity (Barnhart et al., 1980; Francis, 1990). Previous studies of low-level radioactive wastes and waste leachates have shown that microbes in the wastes can metabolize organic carbon compounds (Francis et al., 1980a,b; Francis, 1985). Halotolerant and halophilic microorganisms (10^1 to 10^5 colony forming units/mL) including aerobic, nitrate-reducing, and anaerobic bacteria were detected in the WIPP surficial environment and underground workings (R. Vreeland, West Chester University, Pennsylvania, to be published). Cellulose-degrading extreme halophiles from the underground workings also have been isolated (R. Vreeland, West Chester University, Pennsylvania, to be published). Introduced microorganisms, as well as resident or indigenous halotolerant and halophilic bacteria, can metabolize organic compounds and nitrate in the waste, and may generate metabolic byproducts, such as organic acids, alcohols, carbon dioxide, nitrous oxide, nitrogen, hydrogen, hydrogen sulfide, and methane.

1.2 Biochemistry of Cellulose Degradation

1.2.1 Biodegradation of Cellulose

The cellulosic portion of the TRU waste will approximately be comprised of the following (Brush, 1990): paper (70%), cloth (4%), plywood (10%), and lumber (16%) (untreated: 10% and, treated: 6%). In addition, the waste contains plastic materials (primarily polyethylene and polyvinylchloride) and rubber materials (primarily neoprene and hypalon), the characteristics of which may be altered by alpha-irradiation, which may enhance their biodegradability and potential for gas generation.

Cellulose, hemicellulose, and lignin make up the three major components of plant vascular material, lignocellulose. Lignin is a highly branched, constitutionally undefined aromatic polymer that makes up 15 to 38% of hardwood and softwood trees. It is considered highly resistant to biodegradation, although thermochemically modified lignin has been shown to biodegrade (Colberg and Young, 1982).

Cellulose is an unbranched polymer of several thousand D-glucose units linked together by β-1,4 glucosidic bonds. The strength of the polymer is derived from the multitude of hydrogen bonds, with concentrations of hydrogen bonds in microcrystalline regions and fewer bonds in amorphous regions. Cellulose is insoluble; therefore, hydrolysis is a prerequisite to microbial degradation. Hydrolysis of cellulose results in the formation of cellobiose, which is then hydrolyzed to glucose (Figure 1).

Biodegradation of cellulose by white-rot fungus *Trichoderma reesei* and the bacteria *Cellulomonas* has provided insights into the enzymology, the mechanisms of action, and the pathways of cellulose degradation. Several extracellular enzymes are involved in the breakdown of cellulose. The cellulase enzymes, consisting of exoglucanase (exoenzyme) and endoglucanase (endoenzyme), break the cellulose chains into various smaller fragments, starting with: (i) different 1,4-β-endoglucanases that attack the 1,4-β-linkages, randomly depolymerizing internal units; (ii) 1,4-β-exoglucanases that remove cellobiose from the non-reducing chain end of the molecule, and (iii) 1,4-β-glucosidases (cellobiase) that hydrolyze cellobiose to glucose (Priest, 1984). Amorphous regions of cellulose are degraded by both the endo- and exo-glucanases separately. Synergistic action of the two enzymes is necessary for degrading crystalline cellulose (Poulsen and Peterson, 1992). These enzymes (produced by a variety of aerobic and anaerobic bacteria, fungi, and protozoa) coordinate to hydrolyze cellulose into soluble components, which then are converted into a variety of end products. Bacteria, including aerobes such as *Cellulomonas sp.* and *Cellvibrio gilvus* (Bott and Kaplan, 1991), and anaerobes, such as *Clostridium sp.* (Benoit et al., 1992), *Clostridium thermocellum* (Lynd et al., 1989), *Acetovibrio celluloyticus* (Laube and Martin, 1981), and *Ruminococcus albus* (Pavlostathis et al., 1988) produce extracellular cellulase enzymes in the presence of cellulosics. These enzymes are induced by the presence of substrate (Hrmova et al., 1991) and are attenuated by soil and other absorptive materials (Hope and Burns, 1985). Close proximity of the cell to the substrate is necessary for degradation. A purified enzyme extract of *T. reesei* was shown to effectively degrade cellulose (Priest, 1984), and non-oxygen labile endoglucanase from *Clostridium thermocellum* was shown to strongly absorb to native

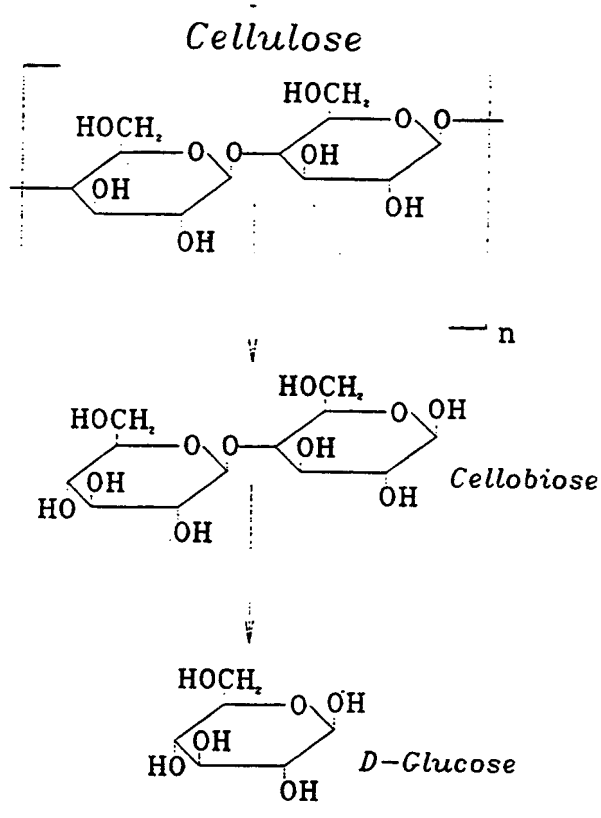

Figure 1. Cellulose Degradation Pathway.

cellulose (Ng et al., 1977). The rate of enzyme induction also depends upon the presence of the necessary nutrients, nitrogen and phosphorus (Skujins, 1976). Upon induction, hydrolysis by the cellulases is the rate-limiting step which, once achieved, follows first-order kinetics (Pavlostathis and Giraldo-Gomez, 1991). The available surface area is an important determinant of the rate of digestion of cellulose. In vitro studies of cellulytic bacteria from cow rumen demonstrated the importance for degradation of adherence of microbial cells to the cellulose surface, with fermentation rates correlated to surface area (Weimer et al., 1990). The primary hydrolysis products of cellulose are cellobiose and glucose, which then are converted to organic acids, carbon dioxide, hydrogen, and methane by various microbial processes.

Glucose, generated from cellulose, is readily used by a variety of microorganisms. The specific process depends on the availability of electron acceptors such as oxygen, nitrate, sulfate, and CO_2. In the presence of oxygen, carbon dioxide and water are formed during the oxidation of glucose:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy}$$

As oxygen is consumed, the alternate electron acceptors are used: nitrate, iron (III) oxides and hydroxides, manganese (IV) oxides and hydroxides, sulfate, and carbon dioxide. In the case of nitrate, dissimilatory reduction transforms nitrate to ammonium (dissimilatory nitrate reduction to ammonium (DNRA)) or to nitrous oxide, and then nitrogen (denitrification):

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+ \text{ (DNRA)}$$

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2 \text{ (Denitrification)}$$

Both processes are affected by the concentration of oxygen; the organisms catalyzing these transformations are microaerophiles or facultative anaerobes, capable of metabolism under low oxygen conditions or in its absence. Denitrification will slow down or cease with higher oxygen concentrations which inhibit the production of specific enzymes (Tiedje, 1988). Denitrifiers use several substrates, such as glucose and low molecular weight organic

acids and alcohols. The use of nitrate as an alternate electron acceptor may be significant in the WIPP because of the presence of nitrate in the waste, predominantly from process sludges.

In the absence of oxygen and nitrate, anaerobic microorganisms will dissipate electrons via fermentation of the carbohydrate:

$$C_6H_{12}O_6 \rightarrow 2C_3H_4O_3 \text{ (pyruvic acid)} + 4H^+$$
$$4H^+ + 2C_3H_4O_3 \rightarrow 2C_3H_6O_3 \text{ (lactic acid)}$$

Fermentation products, such as low molecular-weight organic acids and alcohols are available for preferential use by denitrifiers, sulfate reducers, and methanogens. Brines from the WIPP contain 160 to 300 mM sulfate (Brush, 1990); sulfate-reduction could be significant in the presence of metabolizable carbon. It occurs under reducing conditions (Eh -150 to -200mV, pH = 7.00), resulting in a change in Eh (-250mV) with growth and sulfide formation (Postgate, 1984):

$$2 \text{ lactate}^- + SO_4^{=} \rightarrow 2 \text{ acetate}^- + 2CO_2 + 2H_2O + S^{=}$$

Sulfate reduction results in the formation of H_2S and insoluble metal sulfides. Sulfate-reducing bacteria (SRB) convert lactate, pyruvate, alcohols, amino acids, and acids of the tricarboxylic acid cycle to acetate and CO_2. Glucose and other sugars seldom seem to be used directly by SRB.

The presence of CO_2, H_2, organic acids, and a low Eh generated by these anaerobic microbial processes provide a conducive environment for the growth of methanogenic bacteria. Methanogens can use (i) acetate; (ii) methanol; or (iii) carbon dioxide and hydrogen and produce methane:

(i) $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$

(ii) $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$

(iii) $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

Approximately 70% of the methane formed in sludge and freshwater sediments is due to reaction (i), whereas halophiles predominantly carry out reaction (ii).

Additionally, iron(III) reduction may be a significant process in the WIPP because of the presence of oxidized forms of iron. Iron reduction involves the oxidation of organic carbon concomitant with the reduction of iron, whereby iron is used as the electron acceptor in the absence of oxygen, resulting in the reduction and dissolution of Fe(III) to Fe(II). Manganese reduction results in the formation of soluble Mn(II) from Mn(IV). Soluble uranyl ions can be reduced to insoluble U(IV) by anaerobic bacteria (Francis et al., 1991; Lovely et al., 1992). Corrosion caused by microbes could transform metal ions at a passive surface, resulting in metal sulfide precipitates (Kearns et al., 1992). The use of hydrogen solely from passivation can result in the formation of methane by methanogens, accelerating cathodic depolarization and increasing corrosion (Lorowitz et al., 1992). Figure 2 shows microbiologically mediated redox processes.

1.2.2 Metabolic Diversity of Halophilic Microorganisms

Halobacteria isolated from hypersaline environments can metabolize a wide variety of organic compounds under aerobic and anaerobic conditions. Most of the extreme halophiles are archaebacteria; that is, they are a distinct group of microorganisms, apart from the eubacteria that make up the majority of prokaryotes, with an ancient lineage composed of other types of organisms adapted to extreme environments, such as alkaliphiles, thermophiles, and methanogens. Moderate halophiles grow best in an environment containing 0.5 to 2.5 M NaCl, while extreme halophiles grow best in 2.5 to 5.2 M NaCl (Kushner and Kamekur, 1988; Ventosa, 1988). Brines in the WIPP repository consist of 5.1-5.3 M chloride and 1.83-4 M sodium, 0.63-1.44 M magnesium, and 0.04-0.30 M potassium (Brush, 1990; Molecke, 1983).

Halophiles grow anaerobically by: (i) fermenting glucose, fructose, glycerol, citrate, and lactate (Javor, 1984); (ii) reducing nitrate to nitrogen gas using a variety of carbohydrates

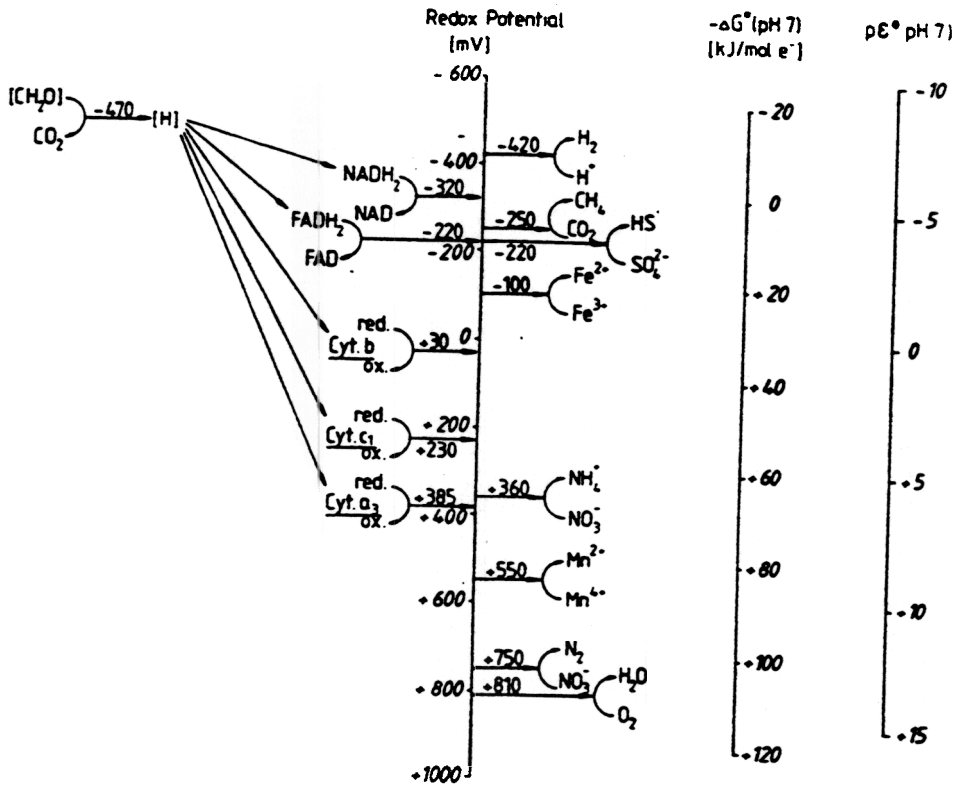

Figure 2. Electron-free energy for biologically mediated redox sequence with organic carbon (CH_2O) acting as the electron donor. Calculated for standard conditions at pH 7. (CH_2O) represents one-sixth of glucose (i.e. -153 kJ mol^{-1}), *from* Zehnder, A.J., and W. Stumm. 1988. "Geochemistry and Biogeochemistry of Anaerobic Habitats," *Biology of Anaerobic Microorganisms*. Ed. A. J. Zehnder. New York, NY: John Wiley and Sons. 19.

(Tomlinson et al., 1986); (iii) degrading chitin; and (iv) producing methane from methylamines, CO_2 and H_2 (Zhilina and Zavarzin, 1990). In hypersaline environments acetate-utilizing methanogens have been difficult to isolate (Zhilina and Zavarzin, 1990). Sulfate reducers also have been difficult to isolate, although saltmarsh sediments harbor abundant SRB populations (Dicker and Smith, 1985). The WIPP site contains a variety of halotolerant and halophilic bacterial populations. Isolates from underground brine seepages and salt crystals, and from brine and sediment from surficial lakes near the WIPP site, revealed a great diversity of colony characteristics when grown on solid media (R. Vreeland, West Chester University, Pennsylvania, to be published); these isolates used amino acids, glucose, and cellulose.

2.0 EXPERIMENTAL APPROACH

Laboratory experiments were designed to determine the potential gas generation due to biodegradation of cellulose under conditions expected in the WIPP repository (Figure The experiments divided into short term 6 months and long-term years ones. In the short-term experiments, we examined the influence of electron donors and acceptors the activities of specific microbial processes relevant to the WIPP disposal nvironment. In the long-term experiments, we measured gas generation due to biodegradation of cellulose under realistic conditions expected in the WIPP repository after the waste was in place. The conditions include humid and inundated, and initially aerobic and anaerobic nvironments. The effects of addition of nutrients and bentonite also was investigated.

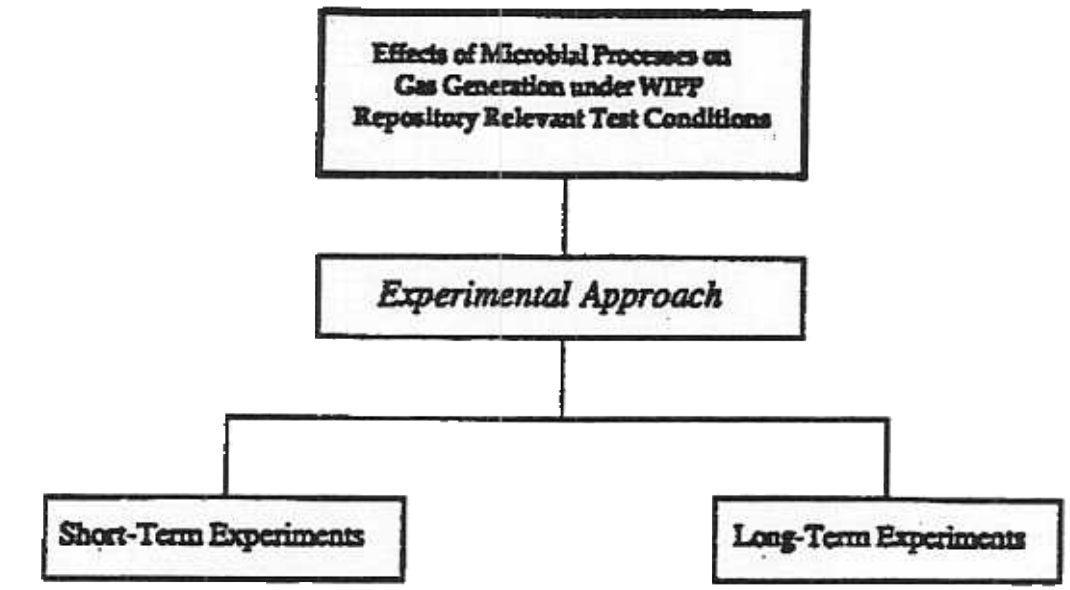

Figure 3. Experimental Approach

3.0 SHORT-TERM EXPERIMENTS

3. Rationale

The two main objectives of the short-term experiments were: to determine gas production due to the activ of aerobes and anaerobes in the presence of specific lectron donors (cellulose, glucose, succinate and acceptors oxygen, nitrate, sulfate under hypersaline conditions; and (ii) to evaluate the suitability of inocula for in the long-term experiments.

Anaerobic microbial processes were emphasized in the short-term experiments because they are expected to have the greatest impact the long term performance of the WIPP repository These experime ts provided opportunity to specify aspects of the long-term investigations by manipulating the experimental variables. The microbes tested include those present in the brine collected from WIPP underground workings, surficial sediment slurries from the surrounding lakes at the WIPP site, and in axeni (pure mixed cultures isolated from these sources. In addition, analytical methods were tested and standardized during this phase

3.2 Methodology

3.2.1 Sample Collection

Sediment and water samples from Nash Draw near the WIPP site and muck pile salt, rib salt, and brine from the WIPP underground workings were collected from August 21 to 25, 1991. Core samples of mud from Laguna Cinco, Quatro, Tres, and Surprise Springs, all in Nash Draw obtained using sterile iron pipes. Air excluded by driving the core deep into the mud and capping it whi submersed. Corrosio of iron end-caps also prevented contamination by oxygen. Lake brine samples were collected in sterile polyethylene containers from the four lakes as well as from Lindsey Lake, also in Nash

Draw. Mud and brine from all the lakes were also collected with sterile glass serum bottles, which were then stoppered and crimped to exclude air. Salt from the WIPP underground was collected using sterile spatulas and sterile containers. G-Seep brine from the WIPP underground was collected in sterile polyethylene bottles by Glen Barker, SNL.

The samples were shipped to BNL within two days after collection. The mud samples were extruded in a nitrogen-filled glove box and transferred to sterile serum bottles, fitted with butyl rubber stoppers, and stored at 4°C. Brine from Lindsey Lake and the WIPP site was also stored at 4°C. Viable bacteria in these samples were counted by Russell Vreeland, West Chester University, and the total number of bacteria and microbial activity were determined at BNL.

3.2.2 Direct Counts of Bacteria

Samples were shaken on a wrist-action shaker for 45 minutes to disperse the contents. One mL was removed with a sterile needle and syringe, dispensed into a snap-cap vial and preserved with 5% (v/v) glutaraldehyde. Double-stranded DNA specific stain 4'6-diamidino-2 phenylindole dihydrochloride (DAPI, Polysciences, Inc.) was added to the sample and it was incubated for seven minutes in the dark. The sample was filtered through a 0.2 μm black membrane filter (Poretics Corp.), and then placed on a slide and examined under oil-immersion at 1875 x magnification. Slides were prepared in triplicate for each sample, the blue-fluorescing cells were counted directly using a calibrated grid eyepiece under ultraviolet light. The DAPI stain differentiated the cells from salt grains: DNA-containing material was blue and the salt yellow (Coleman, 1980).

3.2.3. Activity Measurements

Production of gas by aerobes, anaerobes, and denitrifiers was determined in a mixed inoculum of WIPP salt and brine and Nash Draw brine and sediment. The carbon sources tested included glucose ($C_6H_{12}O_6$), cellulose ($[C_6H_{10}O_5]_n$), and succinate ($C_4H_6O_4$).

Two hundred of S2 80, W30 WIPP muck-pil sal were dissolved in sterile, deionized wate and diluted to L. T 45 mL aliquo of the sol tion, mL of Laguna Cinco mud slurry and 40 mL of Laguna Cinco lake water were added. The mixture was kept in anaerob glove box. Ni and half mL of the mixture were ad ed th sterile syringe to 20 mL steril serum bottles containing .5 mL of sterile, concentrated trient stock solution containing mg east extract, mg potassium phosphate 125 mol ammonium nitrate, and 275 μmol glucose See Appendix A. Aerobic samples were incubated with initial headspace of air in sealed containers, whereas anaerobic sampl were incubated in nitrogen atmosphere afte purging the sampl several tim with nitrogen. The serum bottles were fitted and sealed with butyl rubber stoppers. Denitrification activity determine by adding nutrient solution containing mg yeast extract, mg potassium phosphate, μmol trate added ammonium nitrate, 99 trate added as potassium nitrate, and 85 μmol of succinate. These samples contained total of 224 μmol of nitrate and prepared anaerobically A set of aerobe, anaerobe and denitrifier treatments also prepared by including approximately .5 filter pape instead of the espective carbon sources, glucose and succina

Six samples prepared for each treatment, two of which were eated th mL of 0% formaldehy to serve abiotic controls. Brine samples without nutrients were also epared, and two were treated with formalin to determine ndogenous activity The headspace gas of all the samples analyzed after 48, and 7 days of incubation at 30°C, and the total volume of gas, carbon dioxide, and nitrous oxide in the headspace determined see Appendix B

3.2. Denitrification Studies

In the absence of oxygen and in th presence of metabolizabl organic carbon, some aerobi bacteria can use trate alternate lectron acceptor This rocess, called denitrification, the reduction of nitrate, converts 80% or more of the available nitrate-N

to nitrogen gas (Tiedje, 1988). Nitrate is reduced to nitrogen by denitrifying bacteria via the following steps:

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$$

Nitrate is also reduced by dissimilatory nitrate reduction to ammonium, but with either incomplete conversion to nitrogen or a far lesser yield of nitrogen (Tiedje, 1988). Nitrous oxide is an intermediate product and does not generally accumulate, although some halophiles accumulate nitrous oxide under certain conditions (Tomlinson et al., 1986).

Denitrification was observed in Nash Draw sediment slurry, G-seep, and a pure culture by the acetylene blockage technique (Yoshinari and Knowles, 1976). Acetylene inhibits the conversion (reduction) of N_2O to N_2, resulting in the stoichiometric accumulation of N_2O in the headspace (Balderston et al., 1976):

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O \xrightarrow{C_2H_2} N_2$$

Acetylene was injected into the duplicate samples of each treatment to give a final concentration of 10%. Nitrous oxide in the headspace of each sample was measured with a gas chromatograph equipped with a ^{63}Ni electron capture detector (see Appendix B). Samples were analyzed at 0, 21, and 43 hours, and at appropriate intervals thereafter.

3.2.4.1 DENITRIFICATION IN SEDIMENT SLURRY SAMPLE

Laguna Cinco sediment (collected on August 22, 1991 and stored anoxically at 4°C for two months), and a fresh sample from the same site (collected on December 10, 1991) were examined for denitrifier activity. The fresh sample was examined within 48 hours of collection. The sample was mixed well, and a 1 mL slurry (August: 0.56 ± 0.01 g dry sediment; December: 0.59 ± 0.01 g dry sediment) was pipetted into sterile 20 mL serum bottles in a glove box filled with nitrogen. One mL of filter-sterilized (0.22 μm) brine (20%

/v WIPP halite was added to the slurries resulting in final volume of mL. The slurry contained the following nutrients: (i) additions (unamended) (ii) μmol succinate (carbon-amended); (iii) 300 nmol nitrate (nitrate-amended); and (iv) μmol succinate and 300 nmol nitrate (carbon-and nitrate-amended at a C:N ratio of 0:). Succinate was used as the carbon source to discourage the growth of fermentative organisms. Triplicate samples of each treatment were prepared. The pH of each treatment was measured the beginning and end of the experiment. Formaldehyde-treated samples served as controls. The serum bottles containing samples sealed with rubber stoppers in the glove box in nitrogen atmosphere and incubated at 30°C. Denitrification was determined by the acetylene blockage technique.

3.2.4.2 DENITRIFICATION IN G-SEEP

G-Seep brine collected December 0, 991 from the WIPP underground workings was prepared within 48 hours of collection to assay for denitrification activity Five mL aliquots of G-Seep brine were transferred into sterile serum bottles in anaerobic glove box. One mL of filter-sterilized (0.22 μm brine (20% w/v WIPP halite) with nutrients was added. The G-Seep samples contained the following nutrients: i) no additions (unamended): (ii) 1.5 μmol succinate carbon-amended): iii) 150 nmol nitrate nitrate-amended): and (iv) 1.5 μmol succinate and 150 nmol nitrate carbon- and nitrate-amended). Formaldehyde-treated samples served as controls. Triplicate samples of each treatment were prepared, sealed with butyl rubber stoppers in the glov box in nitrogen atmosphere, and incubated at 30°C. Denitrification was determined by the acetylene blockage technique. Samples were analyzed after 0, 3, 30, and 60 days.

3.3 RESULTS AND DISCUSSION

3.3.1 Bacterial Population

Table 1 shows the numbers of bacteria, both viable and non-viable, in brine samples from Nash Draw and in G-Seep from the WIPP underground. The direct counts of bacteria in Nash Draw samples range from 5.5 x 10^6 to 1.0 x 10^7 cells/mL. The G-Seep brine contained 7.2 x 10^4 to 3.2 x 10^6 cells/mL.

3.3.2 Activity Measurements

3.3.2.1 AEROBIC GLUCOSE METABOLISM

Table 2 shows the total gas, carbon dioxide, and nitrous-oxide production in the mixed inoculum slurry samples incubated initially under aerobic conditions with glucose, ammonium nitrate, yeast extract, and potassium phosphate. In the control samples, there was a slight increase in carbon dioxide due to abiotic reactions, but no nitrous oxide was detected. In amended samples, the concentration of both carbon dioxide and nitrous oxide increased.

3.3.2.2 ANAEROBIC GLUCOSE METABOLISM

Mixed inoculum incubated anaerobically with glucose, ammonium nitrate, yeast extract, and potassium phosphate showed little activity (Table 3). There was no significant production of carbon dioxide in amended samples. The reason for the lack of anaerobic metabolism of glucose in these samples is not known.

Table 1. Bacterial Populations in Nash Draw and G-Seep Samples

Brine Source	Number of Cells/mL
Nash Draw	
Surprise Springs	5.5×10^6
Laguna Cinco	6.8×10^6
Lindsey Lake	7.0×10^6
Laguna Tres South	9.0×10^6
Laguna Quatro	1.0×10^7
WIPP Underground Workings	
G-Seep #9	7.2×10^4
G-Seep #23	3.4×10^6

Table 2. Acrobic glucose metabolism by mixed inoculum.

Treatment*	Incubation Time (Days) 48 Gas Produced** Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	83 Gas Produced** Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	147 Gas Produced** Total (ml)	CO_2 (µmoles)	N_2O (µmoles)
Control (n=2) [Formalin treated]	0.80 ± 0.10	14.0 ± 0.6	nd	1.31 ± 0.57	15.8 ± 0.2	nd	0.79 ± 0.60	20.1 ± 0.1	nd
Amended (n=4)	0.20 ± 0.20	76.9 ± 6.2	7.20 ± 4.58	0.83 ± 0.48	83.0 ± 15.3	8.75 ± 6.14	0.55 ± 0.43	80.4 ± 14.8	8.27 ± 5.71

* Each sample contained 125 µmoles of ammonium nitrate, 278 µmoles of glucose, 5 mg yeast extract, and 10 mg potassium phosphate

** Dissolved gas concentration not included

Table 3. Anaerobic Glucose Metabolism by Mixed Inoculum.

Treatment*	Incubation Time (Days) 48 Gas Produced**			83 Gas Produced**			147 Gas Produced**		
	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)
Control (n–2) [Formalin treated]	0.60 ± 0.50	12.6 ± 0.3	nd	.03 ± 0.23	13.5 ± 0.2	nd	0.13 ± 0.14	16.2 ± 0.1	nd
Amended (n=4)	nd	5.6 ± 0.15	nd	0.34 ± 0.20	7.72 ± 0.18	0.478 ± 0.030	-0.12 ± 0.12	8.00 ± 0.20	2.32 ± 1.59

* Each sample contained 125 µmoles of ammonium nitrate, 278 µmoles of glucose, 5 mg yeast extract, and 10 mg potassium phosphate

** Dissolved gas concentration not included

nd = not detected

3.2.3 CELLULOSE DEGRADATION

3.2.3.1 Aerobic

Table 4 shows llulose degradation in samples incubated tially under aerobic conditions with filter pape nitrate, yeast extract, and potassium phosphate. Control samples showed no activity A significant increase in carbon dioxide was observed after 83 days amended sampl One sample produced 136 μmol of carbon dioxide, and the pressure increased to 4.47 psi. The paper the bottle disintergrated and dissolved. Blackening of specific samples was noted after 14 days, indicative of sulfate reduction.

3.2.3.2 Anaerobic

Samples incubated anaerobically with filter paper ammonium nitrate, east extract, and potassium phosphate showed gnificant increase in ctivity afte 48 days (Table 5). Nitrous oxide was detected in amended samples, bu no in the controls. An increase in carbon dioxide was observed after days. There was large variati in gas production of anaerobi samples; sample produced 103 μmol of carbon dioxide and the pressure reached 12 psi. The filter paper this sampl also disintegrated.

3.2.3.3. Cellulose Degradation the Presence of Excess Nitrate

Mixed inoculum incubated anaerobically with filter paper trate, yeast extract, potassium phosphate, and trate produced nitrous oxide than the succinate-amended samples (Table Only 20% of the nitrate was converted to nitrous de in the samples with acetylene. Carbon dioxide production was no significant until 147 days.

Table 4. Cellulose degradation by mixed inoculum under aerobic conditions.

Treatment*	Incubation Time (Days) 48 Gas Produced**			83 Gas Produced**			147 Gas Produced**		
	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)
Control (n=2) [Formalin treated]	0.90 ± 0.20	10.9 ± 0.5	nd	1.65 ± 0.49	12.7 ± 0.1	nd	1.00 ± 0.43	14.7 ± 0.0	nd
Amended (n=4)**	nd	32.0 ± 1.9	7.29 ± 1.84	0.95 ± 0.61	57.1 ± 22.8	4.12 ± 1.38	nd	63.9 ± 20.7	5.97 ± 2.12

* Each sample contained 125 µmoles of ammonium nitrate, 0.5 grams of filter paper, 5 mg yeast extract, and 10 mg potassium phosphate

** Dissolved gas concentration not included

*** Samples exhibited disintegration of the filter paper at 83 days

nd = not detected

Table 5. Cellulose Degradation by Mixed Inoculum Under Anaerobic Conditions.

Treatment*	Incubation Time (Days) 48 Gas Produced**			83 Gas Produced**			147 Gas Produced**		
	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	Total (ml)	CO_2 (µmoles)	N_2O (µmoles)
Control (n=2) [Formalin treated]	0.60 ± 0.00	11.3 ± 0.1	nd	.02 ± 0.22	12.5 ± 0	nd	0.17 ± 0.17	14.3 ± 0.0	nd
Amended (n=4)									
1	nd	8.85	9.29	0.12	10.7	5.79	0.05	13.1	nd
2***	nd	6.83	10.7	3.48	65.0	nd	.90	95.6	nd
3***	nd	9.12	nd	3.76	103	nd	0.77	90.8	nd
4***	nd	9.73	9.43	2.18	53.0	nd	3.81	105	nd
Mean	nd	8.63 ± 0.54	7.36 ± 2.14	2.39 ± 0.72	57.9 ± 16.5	45 ± 1.25	.63 ± 0.71	76.1 ± 18.4	nd

* Each sample contained 125 µmoles of ammonium nitrate, 0.5 grams of filter paper, 5 mg yeast extract, and 10 mg potassium phosphate

** Dissolved gas concentration not included

*** At 83 days, samples exhibited disintegration of filter paper

nd = not detected

Table 6. Cellulose Degradation by Mixed Inoculum in the Presence of Excess Nitrate.

Treatment*	Incubation Time (Days) 48 Gas Produced**			83 Gas Produced**			147 Gas Produced**		
	Total (ml)	CO_2 (μmoles)	N_2O (μmoles)	Total (ml)	CO_2 (μmoles)	N_2O (μmoles)	Total (ml)	CO_2 (μmoles)	N_2O (μmoles)
Control(n=2' [Formalin treated]									
(w/acetyl.)	nd	8.93	nd	1.03	11.9	nd	0.73	12.7	nd
(w/o acetyl.)	nd	9.90	nd	0.24	10.9	nd	-0.44	12.0	nd
Amended (n=4)									
(w/acetyl., n=2)	nd	5.24 ± 0.40	23.6 ± 1.2	0.38 ± 0.27	20.3 ± 11.4	19.5 ± 2.1	.39 ± 1.97	56.7 ± 36.2	14.4 ± 1.7
(w/o acetyl., n=2)	nd	8.00 ± 0.95	5.59 ± 4.00	0.06 ± 0.02	8.13 ± 0.41	4.94 ± 0.78	3.62 ± 0.88	78.0 ± 5.2	nd

* Each sample contained 224 μmoles of nitrate, 0.5 grams of filter paper, 5 mg yeast extract, and 10 mg potassium phosphate

** Dissolved gas concentration not included

nd = not detected

Cellulose degradation by mixed inoculum was observed in samples incubated under aerobic and anaerobic conditions and in the presence of excess nitrate. Disintegration of the filter paper in aerobic and anaerobic samples was noted after 83 days, indicating cellulose degradation. Analysis of these samples showed an increase in total gas, carbon dioxide, and nitrous-oxide production (Tables 4 and 5). The filter paper exhibited areas of thinning and clearing at 83 days in samples containing excess nitrate. At 147 days, the filter paper had fully disintegrated and carbon dioxide content had increased (Table 6). Aerobic samples containing cellulose showed little increase in carbon dioxide at 147 days; one sample turned black, possibly indicating the onset of sulfate reduction. This sample was checked after 220 days and the presence of hydrogen sulfide was confirmed by gas chromatography. Anaerobic samples also produced hydrogen sulfide at 147 days, and one sample showed blackening (Sample 2, Table 5).

3.3.2.4 DENITRIFICATION

Mixed inoculum samples incubated anaerobically with succinate, ammonium nitrate, yeast extract, potassium phosphate, and excess nitrate exhibited denitrification activity. Complete conversion of nitrate (224 μmol) to nitrous oxide (129 μmol) was noted in the presence of acetylene after 48 days of incubation (Table 7). The accumulation of nitrous oxide in amended samples without acetylene was much less than in the samples containing acetylene, indicating that nitrous oxide was converted to nitrogen. Control samples showed no activity. Additional studies on denitrification by WIPP sediment slurry brine and an axenic culture isolated from the brine are described next.

3.3.3 Denitrification Studies

3.3.3.1 DENITRIFICATION IN SEDIMENT

Microbial denitrification was analyzed in freshly collected sediment and stored sediment. Nitrous oxide was both being produced and converted to N_2 at the same time (acetylene was

Table 7. Denitrification Activity by Mixed Inoculum.

Treatment*	Incubation Time (Days) 48 Gas Produced** Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	83 Gas Produced** Total (ml)	CO_2 (µmoles)	N_2O (µmoles)	147 Gas Produced** Total (ml)	CO_2 (µmoles)	N_2O (µmoles)
Control(n=2; [Formalin treated]									
(w/acetyl.	nd	8.93	nd	.37	10.2	nd	.08	11.8	nd
(w/o acetyl.)	nd	8.26	nd	nd	11.5	nd	nd	12.9	nd
Amended (n=4)									
(w/acetyl., n=2)	nd	3.43 ± 0.24	129 ± 2	0.50 ± 0.20	6.41 ± 0.05	114 ± 5	-0.12 ± 0.24	na	na
(w/o acetyl., n=2)	nd	51.0 ± 36.1	6.97 ± 2.12	1.07 ± 0.20	30.4 ± 21.5	12.1 ± 2.0	1.01 ± 0.39	na	nd

* Each sample contained 224 µmoles of nitrate, 185 µmoles of succinate, 5 mg yeast extract, and 10 mg potassium phosphate

** Dissolved gas concentration not included

nd = not detected

na = not analyzed

not added to the samples) as shown in Figures 4A and 4B. The nitrous oxide concentrations reported in these studies do not include N_2O dissolved in the brine solution. Nitrous oxide was not detected in the control sample, which had been treated with formaldehyde. In unamended samples and samples amended with succinate, ~5 nmol of N_2O was detected at about 45 hours of incubation, and N_2O was not detected in the headspace thereafter. In samples amended with nitrate, the N_2O concentration reached its maximum (30 nmol gdw^{-1}) at about 200 hours and then disappeared rapidly by conversion to N_2 (Figure 4A). In the succinate-and nitrate-amended samples, much less N_2O was detected than in the nitrate-amended samples. Freshly collected samples showed little accumulation of N_2O in the headspace (Figure 4B), most probably due to rapid and complete denitrification.

The rates of denitrification in the unamended and amended (succinate and nitrate) sediment samples were determined by the acetylene blockage technique. Addition of acetylene inhibits the reduction of N_2O to N_2 and allows N_2O to accumulate in the headspace which is analyzed by gas chromatography. In Figures 5A and 5B and Table 8a and 8b, denitrification in stored (2 months) and freshly collected (assayed within 48 hours) samples are compared. In the stored samples, Figure 5A, denitrifying activity initially in the unamended sample was minimal. After a lag phase, denitrification proceeded at about 0.14 nmol N_2O gdw^{-1} h^{-1}. The rate is presented in Table 9a. The sediment contained sufficient indigenous carbon and nitrate to produce 23 nmoles of N_2O gdw^{-1}. Denitrification in the stored, carbon- amended samples was similar to that in the unamended samples (0.22 nmol N_2O gdw^{-1} h^{-1}). Denitrification activity in the stored sample was stimulated by the addition of nitrate. After an initial lag, the rate of denitrification was 1.36 nmol nitrous oxide gdw^{-1} h^{-1}.

A stimulatory effect on the rate and extent of denitrification was seen in both stored and fresh samples when carbon and nitrate were added. Denitrification proceeded at 1.78 nmol N_2O gdw^{-1} h^{-1}, finally producing 209 nmol nitrous oxide gdw^{-1} in the stored sample, translating to denitrification of 80% of the added nitrate. In the freshly collected sediment, without added carbon or nitrate, 205 nmoles of nitrous oxide gdw^{-1} were produced at a rate

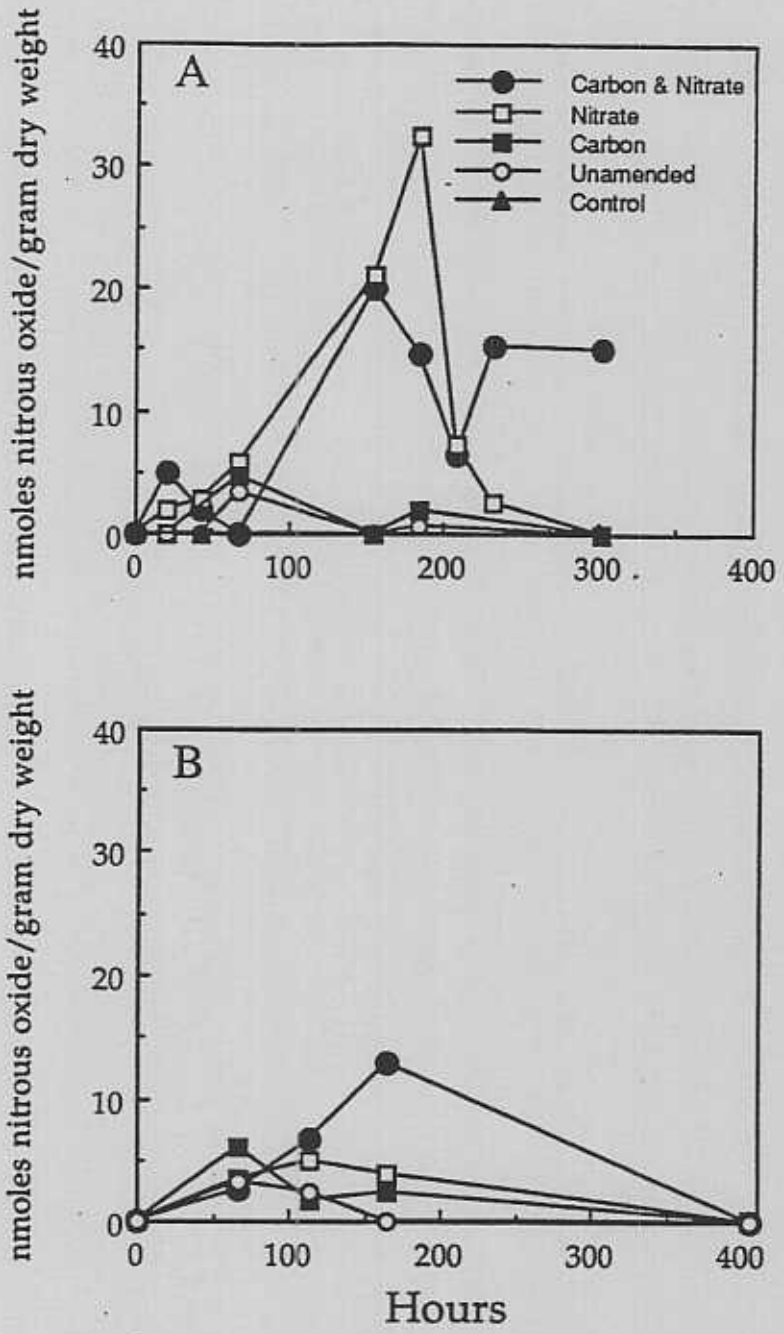

Figure 4. Denitrification by Laguna Cinco sediment; (A) stored sample, (B) fresh sample

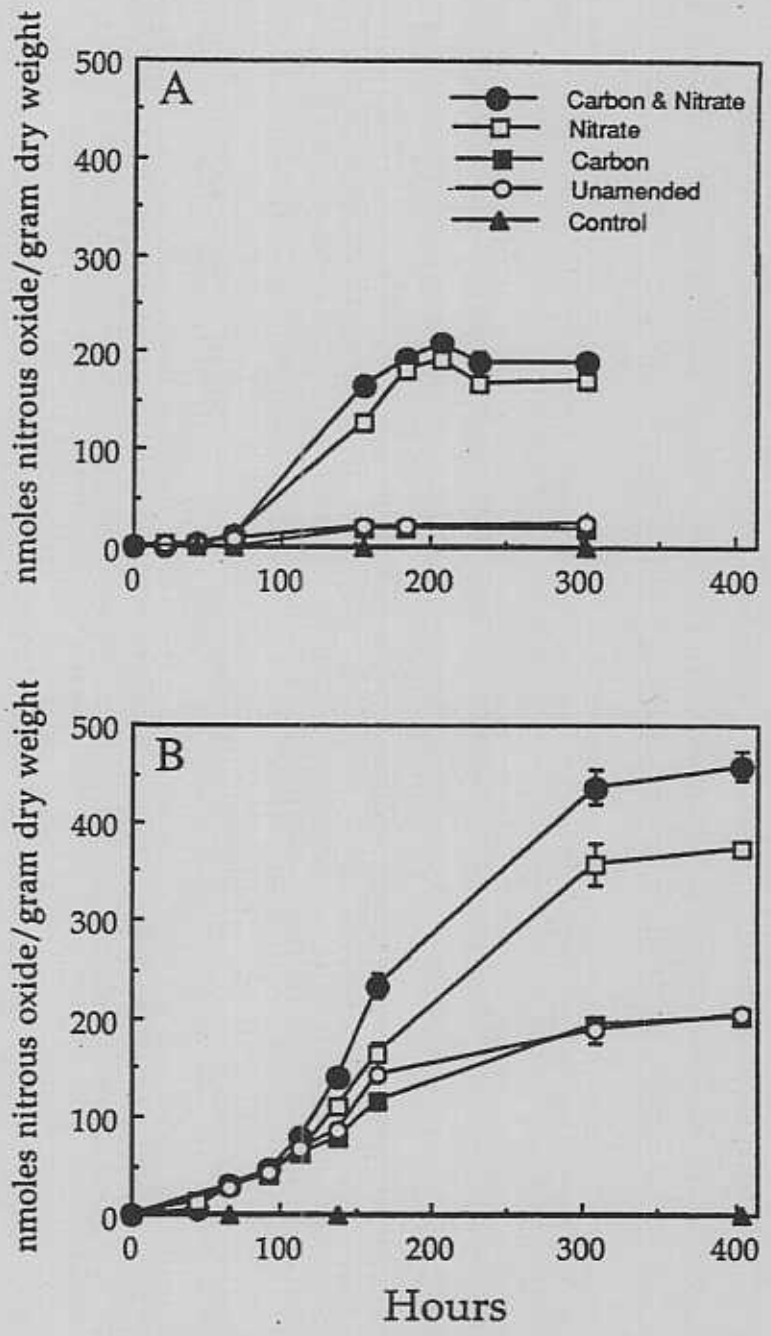

Figure 5. Denitrification determined by the acetylene blockage technique in (A) stored sample, (B) fresh sample

Table 8a. Denitrification in Laguna Cinco Sediment (Stored 2 Months)

Time	Control*	Addition: None	Carbon	Nitrate	Carbon & Nitrate
(Hours)		nmol nitrous oxide**/sample			
0	nd	nd	nd	nd	nd
21		1.96 ± 0.58	2.16	1.50 ± 0.11	0.514
42.5	nd	3.44 ± 0.64	na	3.21	3.86 ± 0.22
67		8.16	0.00 ± 0.00	9.64 ± 0.74	9.64
155	nd	20.7 ± 1.4	19.6 ± 0.1	129 ± 5	166 ± 6
183		22.3 ± 3.0	19.4 ± 0.9	183 ± 0	194 ± 2
207		na	na	192 ± 0	209 ± 2
231		na	na	168 ± 4	191 ± 9
303	nd	23.3 ± 1.5	18.3 ± 0.2	171 ± 8	191 ± 5

Table 8b. Denitrification in Laguna Cinco Sediment (Assayed Within 48 Hours)

Time	Control*	Addition: None	Carbon	Nitrate	Carbon & Nitrate
(Hours)		nmol nitrous oxide**/sample			
0	nd	nd	nd	nd	nd
45		na	na	13.6 ± 0.1	5.58 ± 3.94
66	nd	26.5 ± 1.1	29.0 ± 1.0	30.5	30.5
91.5		43.0 ± 0.3	44.2 ± 4.6	39.4 ± 1.2	45.2 ± 0.5
114	nd	68.6 ± 3.1	61.6 ± 1.8	65.6 ± 2.1	79.6 ± 1.2
138		86.0 ± 3.7	78.1 ± 1.5	110 ± 3	141 ± 5
165		142 ± 5	115 ± 3	164 ± 12	233 ± 12
309		188 ± 11	194 ± 2	357 ± 21	436 ± 18
405	nd	205 ± 1	202 ± 8	372 ± 3	458 ± 15

* Formalin treated samples
** Dissolved gas concentration not included
nd = none detected
na = not analyzed

Table 9a. Denitrification Rate (Stored Sample)*

Addition	Rate (nmol nitrous oxide/g dry weight/hour)
None (Unamended)	0.14
Carbon	0.22
Nitrate	1.36
Carbon and Nitrate	1.78

*Rate after lag phase, from 67 to 155 hours (see Table 8a).

Table 9b. Denitrification Rate (Fresh Sample)*

Addition	Rate (nmol nitrous oxide/g dry weight/hour)
None (Unamended)	1.17
Carbon	0.87
Nitrate	1.93
Carbon and Nitrate	3.00

*Rate after lag phase, from 66 to 165 hours (see Table 8b).

of 1.44 nmol nitrous oxide gdw^{-1} h^{-1}, as shown in Table 9b. These samples are not carbon-limited. Addition of nitrate, however, increased N_2O production, suggesting that these samples are nitrate-limited. In the absence of carbon or nitrate limitations in the freshly collected sediment, denitrification proceeded at 3.00 nmol N_2O gdw^{-1} h^{-1}, and resulted in the production of 458 nmol nitrous oxide gdw^{-1} (Table 8b); all of the total nitrate present was reduced.

These experiments show that microorganisms present in mud from Laguna Cinco denitrified 70-80% of the added nitrate in nitrate-amended samples and 80%-100% of the nitrate in carbon and nitrate samples. We added 540 nmol nitrate gdw^{-1} to each sample; therefore, complete conversion of the nitrate to nitrous oxide should yield 270 nmol N_2O gdw^{-1}. About 70% of the added nitrate was reduced to nitrous oxide in the stored sample, or 192 nmoles N_2O gdw^{-1} was produced. Freshly collected sediment with added nitrate produced 372 nmoles N_2O gdw^{-1}; 81% of the total nitrate present was reduced. This sediment contained a large quantity of indigenous nitrate.

Denitrification in unamended samples provides information about the nutrient conditions in the sediment. If all the available indigenous nitrate was converted to nitrous oxide in the carbon-amended sample, then the stored sediment contained about 40 nmoles of nitrate gdw^{-1}, and the fresh sediment contained 410 nmol gdw^{-1}. Similar conversions of nitrate to nitrous oxide using a pure culture were used to determine sub-ppb concentrations of nitrate in lake waters (Christensen and Tiedje, 1988). Thus, the assay can be used not only to detect the presence and activities of denitrifying organisms, but also to detect easily metabolizable low-molecular-weight organic carbon compounds in the environment (Francis et al., 1989).

The presence of metabolizable carbon and nitrogen compounds in the WIPP surficial environments has important implications. For example, a steady mix of microbial populations can be actively maintained when presented with an adequate supply of a

limiting nutrient. From the standpoint of the long-term experiment, we have shown, in part, that the organisms are active in the proposed inoculum.

The results also show that active denitrification under anaerobic conditions occurred in Laguna Cinco mud collected in August 1991 and stored for two months, and in mud collected in December 1991, and assayed within 48 hours. Therefore, the stored and fresh samples contain viable organisms that can be used as an inoculum for the long-term experiment.

3.3.3.2 DENITRIFICATION IN G-SEEP

Figure 6 and Table 10 show denitrification in G-Seep brine. In the carbon and nitrogen amended samples ~ 84% of the added nitrate (150 nmoles) was converted to N_2O (62.7 nmoles) after two months of incubation. The dissolved N_2O in the brine was not determined; hence, the N_2O values reported are not corrected for N_2 solubility. Lack of N_2O production in the unamended and carbon amended samples suggests that G-Seep brine is nitrate-limited. However, in the nitrate-amended samples, 45% of the added nitrate was converted to N_2O, indicating metabolizable carbon is present which eventually became limiting. These results also suggest that the microbes in the brine were able to readily metabolize the added carbon and nitrate via denitrification.

3.3.3.3 DENITRIFICATION BY AN AXENIC PURE CULTURE

A pure culture of a denitrifying bacterium was isolated from the sediment slurry sample during the initial denitrification experiments. The isolate was grown in the following medium: sodium succinate, 5 g; potassium nitrate, 1 g; yeast extract, 0.5 g; WIPP salt (20% w/v), 1000 mL; pH 6.85. The isolate, designated as BWFG-1, was a gram-negative rod, facultative anaerobe, and grew rapidly within 48 hours in the liquid medium. On solid medium, the culture produced circular, convex, light-orange colonies with entire margins.

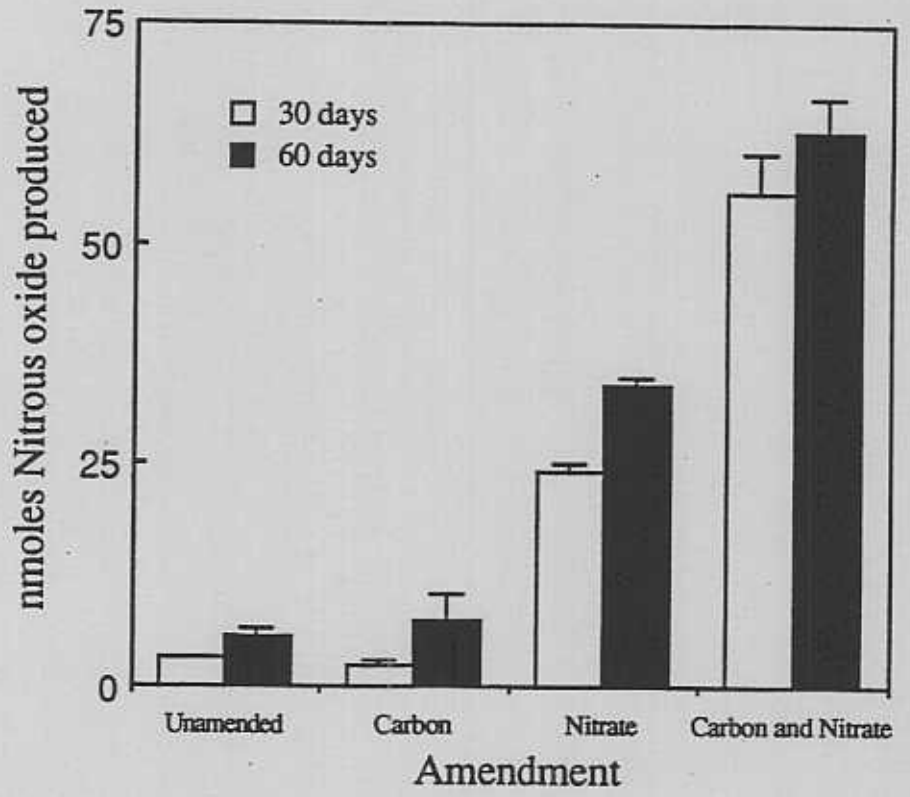

Figure 6. Denitrification in G-Seep Brine. [In C&N amended samples, 84% of the added nitrate was converted to nitrous oxide]

Table 10. Denitrification in G-Seep Brine

	Amendment*				
Time	Control*	Unamended	Carbon	Nitrate	Carbon & Nitrate
(Days)	nmoles nitrous oxide***/sample				
0	nd	nd	nd	nd	nd
3.25	nd	nd	nd	nd	nd
30	nd	3.30 ± 0.01	2.53 ± 0.43	24.2 ± 0.8	55.9 ± 4.4
60	nd	5.72 ± 0.72	7.58 ± 2.98	34.0 ± 0.9	62.7 ± 4.0

Samples injected with acetylene to accumulate nitrous oxide
* Amendment: Carbon = 1.5 umol succinate, Nitrate = 150 nmol
** Formalin treated samples
*** Dissolved gas concentration not included
nd = none detected

BWFG-1 is an archaebacterium. This was confirmed by hybridization with fluorescently labeled oligodeoxynucleotide probes complementary to the 16S ribosomal RNA segment specific for archaebacteria (DeLong et al., 1989). Extreme halophiles were previously described in the literature as being archaebacteria, placing them in a distinct phylogenetic group of organisms that contains other genera from extreme environments, such as methanogens, thermoacidophiles, and alkalophiles (Ross et al., 1981).

The rate of denitrification by BWFG-1 was determined by adding 2.5 mL of a 24 h culture to 40 mL of medium in the presence of acetylene. Control samples included uninoculated medium, inoculated samples treated with 0.5% v/v formaldehyde, and inoculated samples without acetylene. Triplicate samples were incubated anaerobically at 30°C. The number of bacterial cells were counted using the DAPI method.

Production of nitrous oxide by the pure culture is shown in Figure 7 and Table 11. The bacterium denitrified nitrate at a rate 2.5 μmol h^{-1}. About 72% of the added nitrate (392 μmol) was converted to N_2O (142 μmol) in about three days; these N_2O values do not include the amount of N_2O dissolved in the growth medium. There was no accumulation of nitrous oxide in samples incubated without acetylene, indicating complete reduction of nitrate to N_2. In Figure 8, the direct counts of the bacteria during the course of denitrification are presented. A marked increase in the number of cells corresponding to nitrous oxide production was observed. The pH of the growth medium increased from 6.85 to 8.00 after three days, and turbidity measured spectrophotometrically at 600 nm was 0.08 at 0h, 0.58 at 29h, and 0.80 at 50h.

3.4 Summary, Short-Term Experiments

1. Direct microscopic examination of brine from Nash Draw lakes and from G-Seep showed that between 10^4 to 10^7 cells/mL bacteria are present.

2. Cellulose was degraded by a mixed culture derived from samples consisting of Nash Draw sediment slurry, salt crystals, and G-Seep brine in the presence of added nutrients (nitrate, phosphate, and yeast extract). Cellulose degradation was confirmed by an increase in carbon dioxide production and disintegration of filter paper.

3. Storage of sediment and lake water at 4°C for about two months did not significantly affect the activity of microbes in the samples.

4. The denitrification assay is a useful method to rapidly determine the activity of dentrifying microbes in WIPP samples. The assay also confirmed the presence of metabolizable carbon in the sediment and in WIPP brine.

5. Denitrifiers were detected in G-Seep, although their source was not identified.

6. An axenic culture of archaebacteria was isolated from the WIPP site and denitrified nitrate at a rate of 2.5 μmol h^{-1}. The characteristics and growth rate of this culture have been elucidated for future studies to examine the influence of environmental variables on specific microbial processes in the WIPP repository.

7. Short-term experiments have provided useful information on microbial activity under accelerated test conditions that are relevant to the WIPP repository; further work will include examination of the other anaerobic processes such as fermentation, sulfate reduction, and methanogenesis.

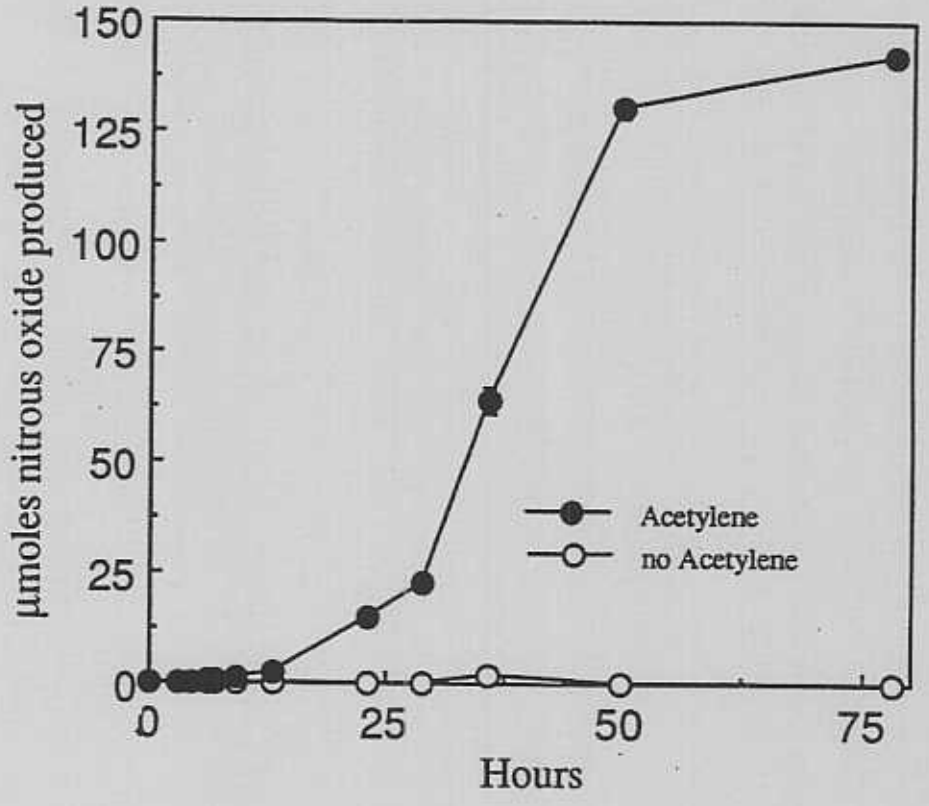

Figure 7. Denitrification by a pure culture of bacteria isolated from the WIPP site. [72% of added nitrate was converted to nitrous oxide at a rate of 2.5 µmoles/h]

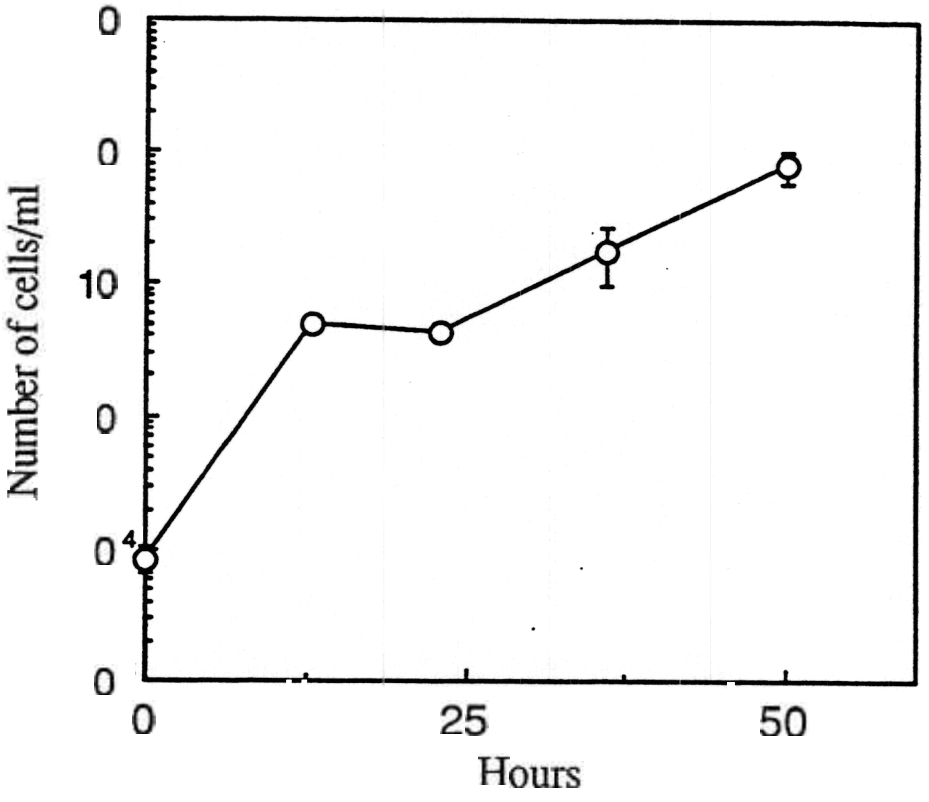

Figure 8. Growth of denitrifying bacteria.

Table 11. Denitrification by a Pure Culture Isolated from the WIPP Environment: Nitrous Oxide Production Over Time

Time (Hours)	Inoculated (w/o acetylene)	Inoculated (w/ acetlyene)	Inoc.* (w/o acet.)	Inoc.* (w/acet.)	Uninoc. (w/o acet.)	Uninoc. (w/acet.)
	----------µmoles nitrous oxide**/sample----------					
0	nd	nd	nd	nd	nd	nd
3	0.001 ± 0.001	0.011 ± 0.034	nd	nd	nd	nd
4.5	0.007 ± 0.002	0.245 ± 0.081	nd	nd	nd	nd
6	0.009 ± 0.002	0.434 ± 0.090	nd	nd	nd	nd
7	0.010 ± 0.003	0.563 ± 0.016	nd	nd	nd	nd
9.25	0.009 ± 0.003	0.990 ± 0.027	nd	nd	nd	nd
13	0.014 ± 0.003	2.33 ± 0.03	nd	nd	nd	nd
23	0.019 ± 0.003	15.2 ± 0.9	nd	nd	nd	nd
29	0.187 ± 0.084	23.0 ± 0.5	nd	nd	nd	nd
36	1.50 ± 0.38	63.8 ± 2.6	nd	nd	nd	nd
50	0.086 ± 0.059	130 ± 2	nd	nd	nd	nd
78	0.140 ± 0.030	142*** ± 1	nd	nd	nd	nd

Defined culture medium (per sample): 740 umoles succinate, 392 umoles nitrate, 20% w/v WIPP salt.
* = Sample treated with formalin (0.5%)
** = Dissolved gas concentration not included
*** = 72% of nitrate added converted to nitrous oxide
ND = none detected

4.0 LONG-TERM EXPERIMENTS

4.1 Objective

The objective of the long-term study is to determine the rate and extent of gas generation over the long term (>2 years) from cellulose biodegradation under humid and inundated conditions, in the presence and absence of added nutrients.

4.2 Rationale

The TRU waste that will be placed in the WIPP repository contains an average of about 10 kg of cellulosic material per drum, approximately 70% of which is paper (Brush 1990). Initially, the repository will be ventilated, but the addition of backfill (salt, or bentonite/salt mixture to fill void spaces around waste containers) will seal the drums inside the disposal rooms. Initially, the repository will also be dry, but after sealing, humid conditions will develop. The ambient humidity is expected to be 18 to 27 g/m^3 (about 74% relative humidity, RH), and the temperature about 30°C (Brush, 1990). Microenvironments of condensed liquid brine may exist under humid conditions. Diffusion of water vapor through high-efficiency particulate (HEPA) air filters on waste containers will result in humid conditions inside the containers. Eventually, corrosion or rupturing of the containers due to salt creep-room closure will expose the waste to salt and backfill. Process sludges from other breached waste containers are expected to be leached by the brine. This is presumed to be the major source of nitrate and phosphate (nutrients) in the repository. The accumulation of potentially intruding brines from the surrounding Salado Formation will most likely begin after sealing the rooms, which will inundate them. In the event of potential, inadvertent human intrusion, fluids may also seep in from the Castille Formation into the Salado Formation. The atmosphere inside the disposal environment will become anaerobic in the short-term (months to years) due to consumption of oxygen by corrosion, radiolysis, and microbial processes acting on the waste materials. Microenvironments of trapped air that contain oxygen will continue to exist after sealing. Radiolysis of organic

wastes will deplete oxygen, whereas radiolysis of nitrate-bearing sludges will release oxygen. Radiolysis of brines may also produce some oxygen.

A succession of microbial processes will occur under the changing ironmental conditions inside the epository Environment changes from bi to anaerobic, humid to inundated and possibly back to humid and asaline to saline will affect th activities of (i) crobes initially presen in the waste, and resid and indigenous halotolerant or halophilic bacteria the brine and sal To examine the influences of various microbial processes on gas ge ration, samples were treated to simulate the following scenarios.

4.2. Aerobic (Sealed) Treatments

During the early stages of waste emplacement, the environment will be aerobic but will become anaerobi time after closure because of corrosion, aerobic microbial ictivity and radiolytic processes. In these long-term experiments, the cellulose samples will be placed in serum bottles, sealed th air and incubated. Th conditions will be initially aerobic and become anaerobic with time due to consumption of oxygen by aerobes, thus paving the way for anaerobi microbial activity

›.2. SCENARIO

After emplacement and sealing of waste containers in WIPP disposal rooms, the intact and nearly intact containers will be isolated from backfill and brine. The humidity inside the disposal rooms is expected to be to 27 g/m^3 about 74% RH) and humidity inside the containers is expected to 'each equilibrium th the room environment. The cellulose will be asaline, humid, aerobic environment for possibly months, years, up to few decades, ter vapor diffuses through the waste drum particulate filters. Microorganisms capabl of cellulose degradation and gas production under these conditions will be active probably environmen th sub-optimal moisture content.

4.2.1.2 SCENARIO 2

Room closure and corrosion will breach many of the containers and expose the waste material to backfill, salt, and brine. The cellulose is expected to contact the salt and backfill material, and microbial degradation of the cellulose is expected to occur under saline, humid conditions.

4.2.1.3 SCENARIO 3

Influx of intruding brines from the Salado Formation, capillary rise through the backfill, and dissolution of brine will all tend to inundate some portion or all of the disposal rooms with brine. Inundation will accelerate the onset of anoxic conditions as any residual air pockets are flooded. Process sludge TRU wastes contain significant quantities of nitrate and lesser quantities of phosphate. The breach of these sludge containers and inundation by brine will then transport the nitrate and bring nonhalophilic, halotolerant and halophilic microbes into contact with cellulose.

Inundation of the WIPP waste by brine by the above or other processes will accelerate the activities of halophilic and halotelerant microbes. In particular, dentrification activity under microaerophilic and anaerobic conditions could be significant and may contribute to the total quantity and to the proportion of gases produced (N_2, N_2O, and CO_2).

4.2.2 Anaerobic Treatments

At least a portion of the WIPP repository wastes will be anaerobic at the start (within their containers possibly due to radiolysis and microbial action at the initial stages) and remain anaerobic thereafter. Under these conditions, short-term (i.e., operational phase) and long-term degradation of cellulosic waste by anaerobic microorganisms could be significant.

4.2.2.1 SCENARIO 4

Some of the cellulose in the disposal environment may become anaerobic before any significant aerobic microbial activity. Cessation of air flow from closure of the disposal rooms, and oxic corrosion plus radiolysis, may bring about anoxic conditions in a humid environment. If the cellulose is exposed to salt under humid conditions, halotolerant or halophilic microbes that can grow in humid and anoxic environments may be involved in degrading cellulose. With the onset of anoxic conditions, alternate electron acceptors such as nitrate and sulfate will be used by microbes in degrading cellulose and its degradation product intermediates.

4.2.2.2 SCENARIO 5

With the onset of brine intrusion in the disposal rooms, inundation will be more likely to cause anaerobiosis by forcing out any residual trapped air. Cellulose in contact with brine may undergo degradation by halophilic and halotolerant microbes present in the brine and waste. Because of the breaching of the waste containers, it is likely that nitrate originating in the sludges will be transported by the brine. It may come in contact with cellulosic wastes and enhance the degradation of cellulose.

Scenarios 3 and 5 will be examined in the long-term inundated experiment. Scenarios 1, 2 and 4 will be examined in a long-term humid experiment in CY1993. Figures 9 through 12 give the complete treatment matrix for the long-term inundated experiments.

4.3 Materials and Methods

4.3.1 Cellulosics

Simulated TRU cellulosic waste material was composed of four types of paper: (i) filter paper, (ii) white paper towel, (iii) brown paper towel, and (iv) Kimwipes® (lintless tissue

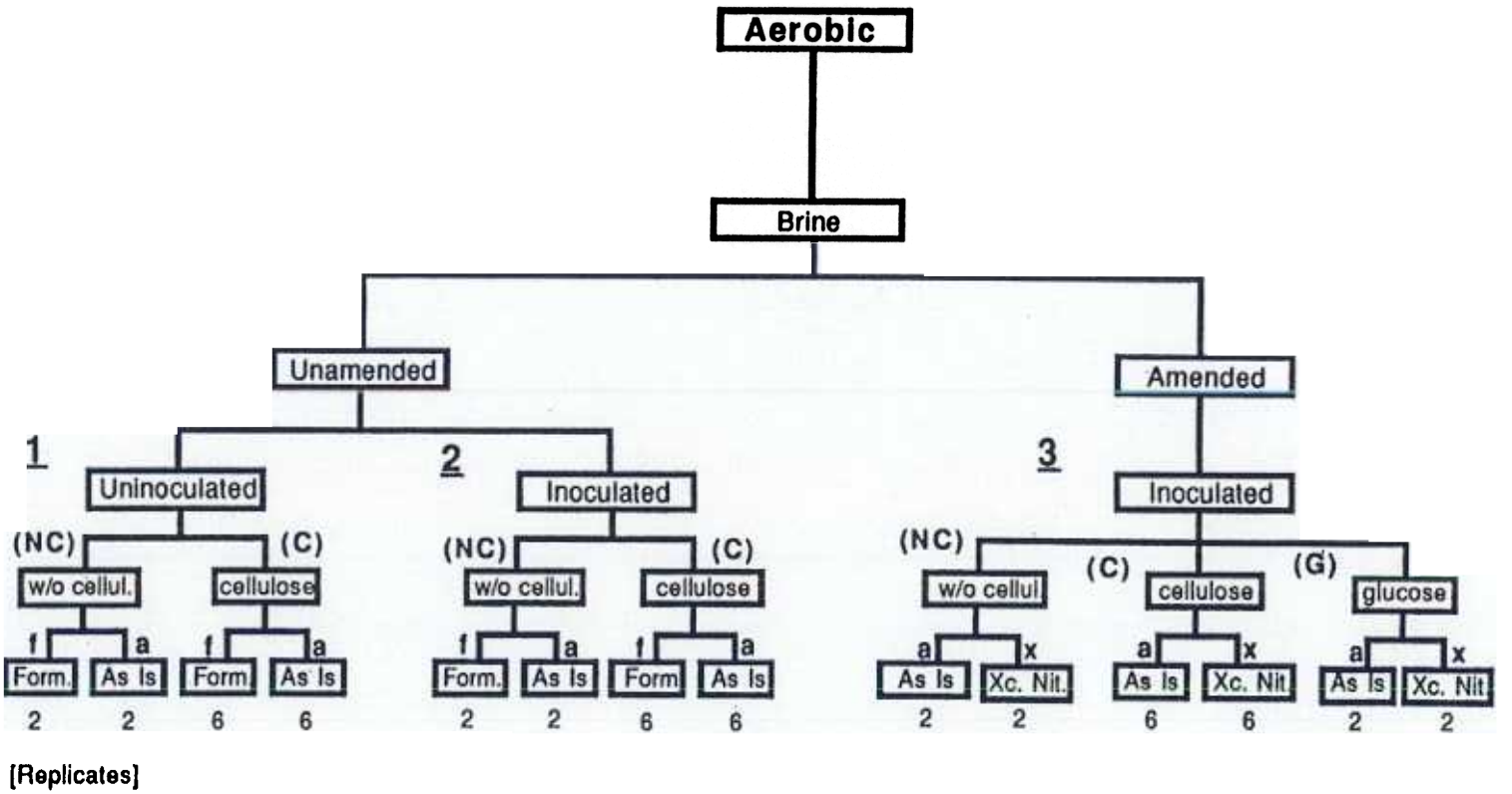

Figure 9. Long-term inundated experiment treatment matrix (aerobic samples).

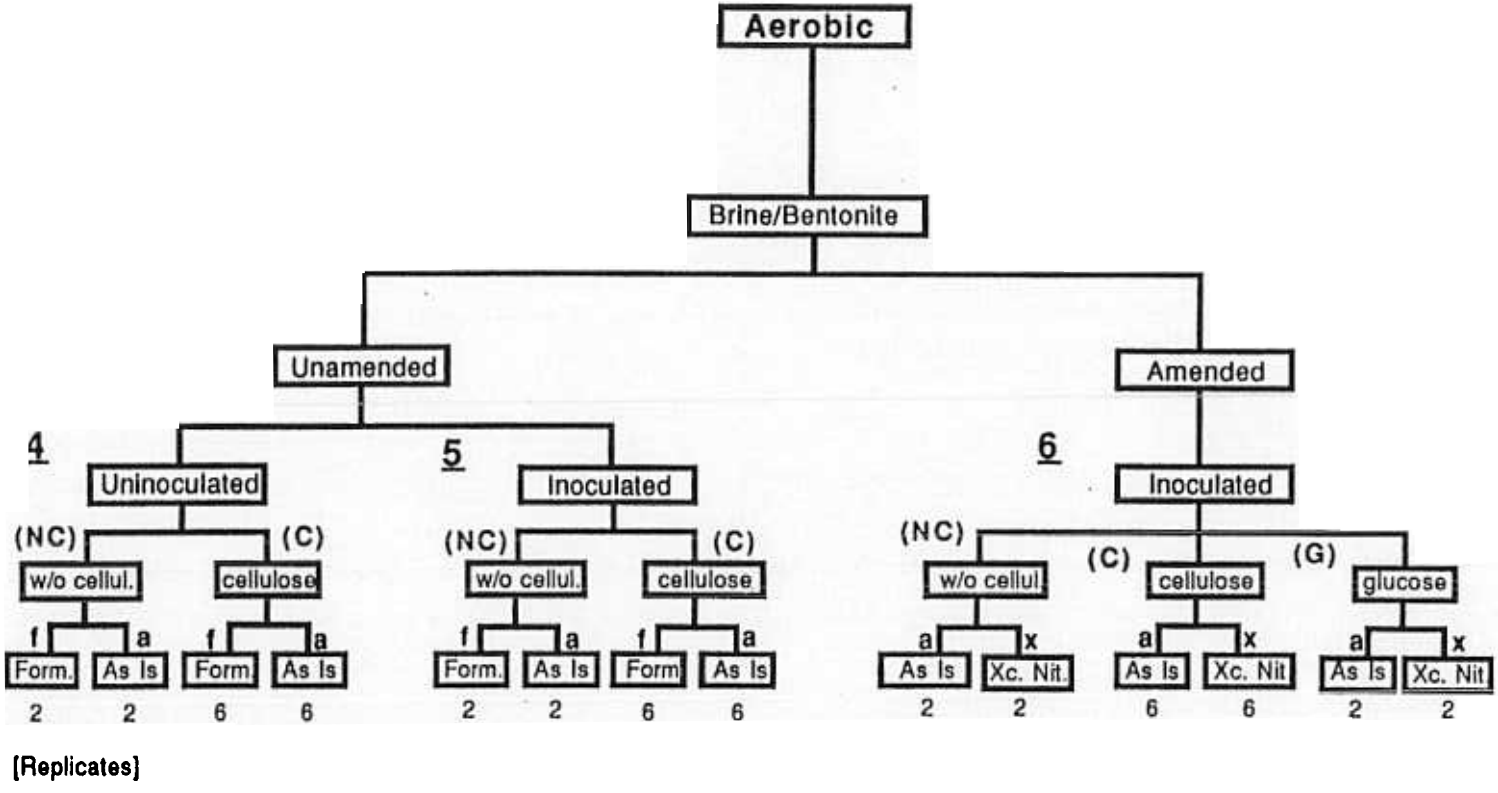

Figure 10. Long-term inundated experiment treatment matrix (aerobic samples containing bentonite).

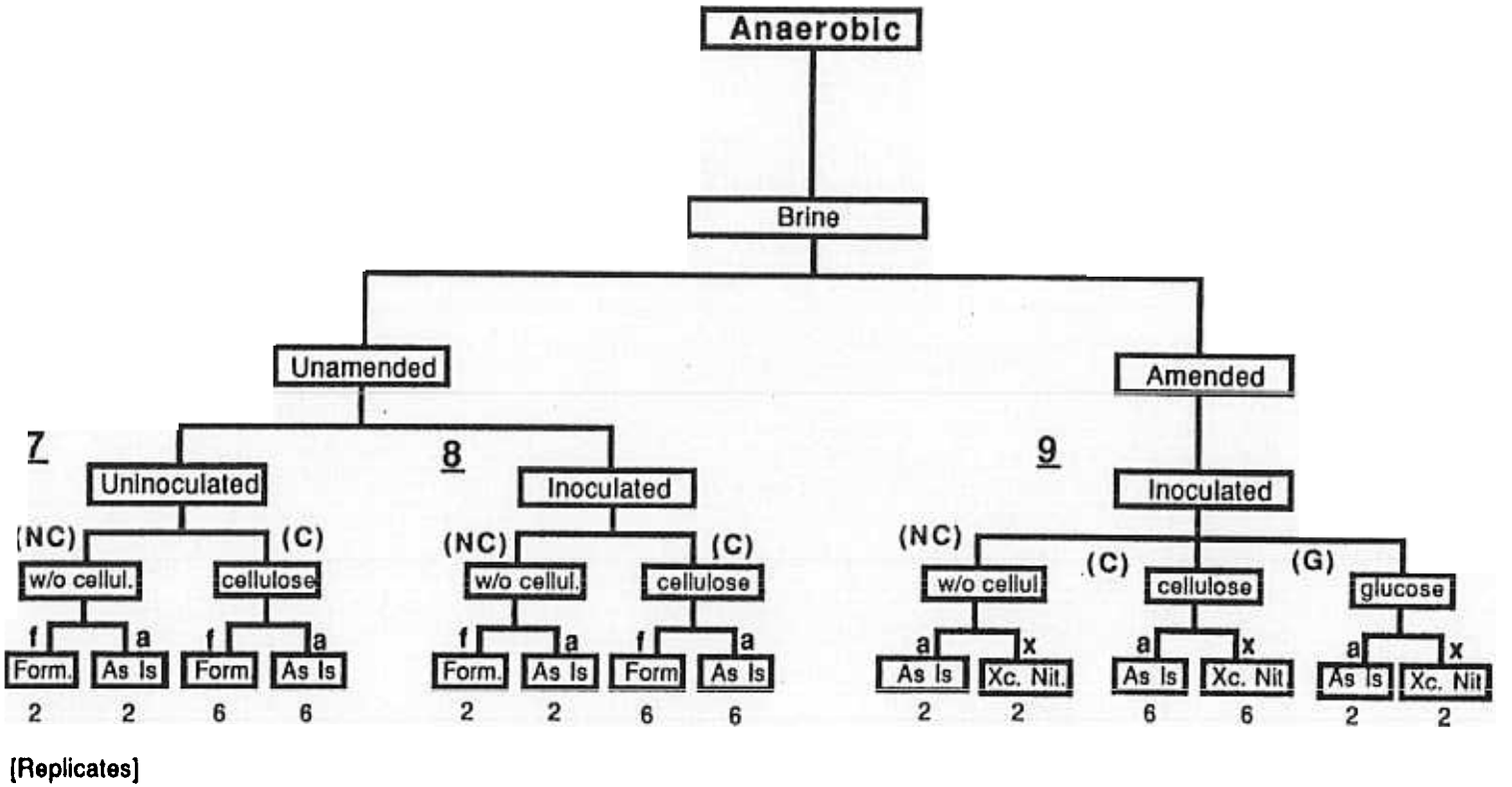

Figure 11. Long-term inundated experiment treatment matrix (anaerobic samples).

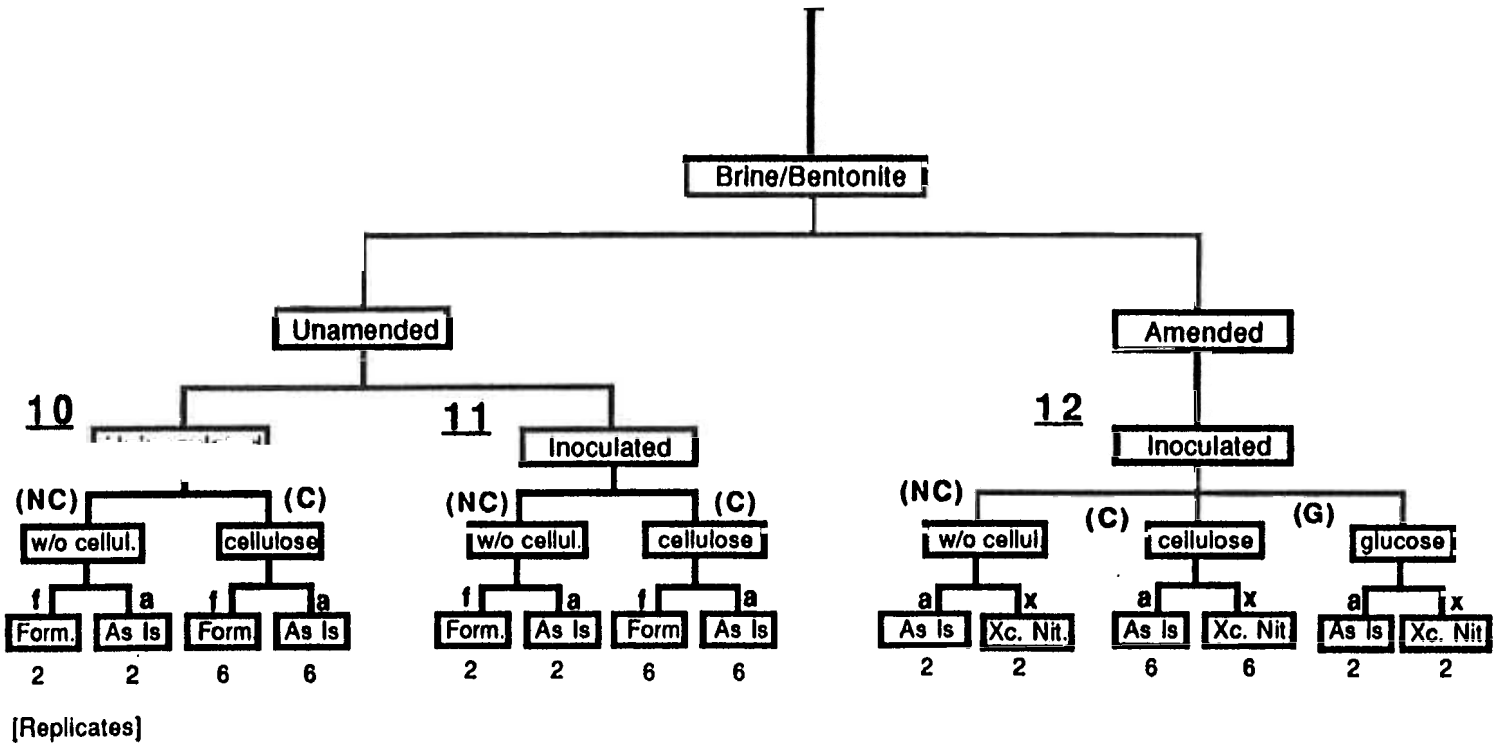

Figure 12. Long-term inundated experiment treatment matrix (anaerobic samples containing bentonite).

wipes). These papers are typical cellulosic wastes from laboratory and process activities. The four paper types were cut into strips in a large paper shredder, and then cut into 1 cm x 1 cm squares.

Samples of shredded paper types, each weighing 1.25 g, were thoroughly mixed and added to 160 mL acid washed (10% HCl), rinsed, sterile (autoclaved at 120°C, 20 psi for 20 min) serum bottles.

4.3.2 WIPP Brine

Fifteen liters of G-Seep #9 brine were provided by SNL and stored at 4°C until use.

4.3.3 Bentonite

Bentonite clay in two one-L containers was provided by SNL. The bentonite was a granular MX-80 Volclay bentonite available from the American Colloid Company of Belle Fourche, SD (Table 12).

4.3.4 Inoculum

The microbial inoculum used in these studies were obtained from the following three sources (Table 13): (i) mud and brine from Nash Draw: collected on December 12, 1991 and stored at 4°C, the mud was stored anoxically in serum bottles, (mud samples were filtered through sterile cotton in a nitrogen-filled glove box to remove large particulates); (ii) brine from the WIPP underground workings: 200 mL of G-Seep were collected on December 12, 1991; and (iii) asaline inoculum from laboratory contamination: 2.5 g of dust containing asaline microorganisms was gathered from laboratories in Bldg. 318 at BNL.

The mud, brine, and dust samples were then mixed together in a sterile beaker in a nitrogen-filled glove box. The total volume of the mixed inoculum was 583 mL. The

Table 12. Composition of Bentonite*.

Chemical Composition	$(NaCa)_{.35}$ $(Al_{1.60}Fe_{.15}Mg_{.25})$ $(Si_{3.90}Al_{.10})$ 0_{10} $(OH)_2$		
Montmorillonite Content	90 Percent		
Typical Chemical Analysis	Silica		63.02% SiO_2
	Alumina		21.08% Al_2O_3
	Iron (Ferric)		3.25% Fe_2O_3
	Iron (Ferrous)		0.35% FeO
	Magnesium		2.67% MgO
	Sodium and Potassium		2.57% Na_2O
	Calcium		0.67% CaO
	Crystal Water		5.64% H_2O
	Trace Elements		0.72%
Exchangeable Ions (Milli-equivalents/ 100 g)	Sodium	55-65	
	Calcium	15-25	
	Magnesium	10-15	
Moisture Content	10% Maximum as Shipped		
pH	8.5 - 10.5		

* Data provided by the American Colloid Company, Skokie, IL.

Table 13. Composition of Mixed Inoculum.

Source	Mud Slurry (mL)	Brine (mL)
Laguna Quatro Mud and Brine	60	40
Laguna Cinco Mud and Brine	35	40
Laguna Tres South Mud and Brine	13	40
Lindsey Lake Mud and Brine	50	40
Surprise Springs Mud and Brine	25	40
G-Seep Brine		200
Total	183	400

activity of the mixed inoculum was examined by incubation under aerobic and anaerobic conditions in the presence of metabolizabled substrate. The results are presented in Appendix E.

4.4 Sample Treatments

The treatments consisted of (a) 100 mL of brine, and (b) 100 mL of brine and 5 g mixed cellulosic papers. The samples were incubated with and without nutrients, which consisted of yeast extract (0.05%), potassium phosphate dibasic (0.1%), and ammonium nitrate (0.1%). Some samples also received excess nitrate as potassium nitrate (0.5%).

4.4.1 Anaerobic Sample Preparation

The serum bottles containing the mixed cellulosic papers were flushed with nitrogen and placed inside an anaerobic, nitrogen-containing glove box for 24 hours before inoculation to remove any trapped air. G-Seep brine (10 L) was removed from storage at 4°C and equilibrated overnight at room temperature. One hundred mL of the brine solutions (with and without nutrients or excess nitrate) were added to sample bottles with and without bentonite containing either no cellulose, cellulose, or glucose. Bentonite (6 g) was added to separate sample bottles inside the glove box to determine its influence on gas production. The samples were gently mixed to distribute the bentonite.

The microbial inoculum prepared from various sources was continually mixed and 4 mL was added to specific samples (3.8% V/V inoculum). The samples were gently mixed (to blend the inoculum) and then capped with butyl rubber stoppers. Control samples received 3 mL of 37% formaldehyde to give a final concentration of 1% formaldehyde.

4.4.2 Aerobic Sample Preparation

Aerobic (sealed) samples were prepared as described above with the following exceptions: 1) brine solutions not purged with ultra high-purity (UHP) N. the mixed inoculum was removed from the glove box; brine was added to the bottles, inoculated, and capped with butyl rubber stoppers outside the glove box, thereby sealing air in the headspace. Appendix C has detailed description of all the treatments (aerobic and anaerobic) and the number of replicate samples. All samples were placed in 30 ± 2°C incubator

4.4.3 Gas Analyses

The headspace gas of select samples was analyzed for total gas production, carbon dioxide, and nitrous oxide at time 0 (January 29 992 and thereafter at monthly intervals. Control samples analyzed less frequently The methods used for the headspace gas analyses are presented in Appendix B.

5.0 RESULTS AND DISCUSSION

The treatments consist of cellulose samples which were (i) uninoculated, (ii) inoculated with a mixed inoculum, (iii) inoculated and amended with nutrients, (yeast extract (0.05%), potassium phosphate (0.1%), and ammonium nitrate (0.1%)), and (iv) inoculated with nutrients plus excess nitrate (0.5% potassium nitrate).

The results presented for aerobic and anaerobic samples represent the amount of gas produced per gram of cellulose plus or minus 1 standard error of the mean (Figures 13 through 24) . A detailed description of the procedure used to calculate the results are given in Appendix D. Tables 1 through 12 in Appendix D present data on a per sample basis, and Tables 13 through 24 in Appendix D present data on a per gram cellulose basis. Gas production rates on a per gram cellulose per day basis are presented in this section in Table 14, and on a per drum of waste per year basis in Table 15.

5.1 Aerobic Treatments

5.1.1 Total Gas Production

Figure 13 shows the total gas produced in samples incubated with an initial atmosphere of air (aerobic). The formalin-treated control samples showed no increase in total gas production, and, in fact, showed a slight decrease. Likewise, uninoculated and inoculated samples which received no nutrients showed a slight decrease in total gas production (-0.18 mL g^{-1} cellulose and -0.34 mL g^{-1} cellulose respectively (Table 13, Appendix D)). The decrease in total gas may be due, in part, to sampling. A decrease in gas production was more evident in inoculated samples because of oxygen consumption, indicating the start of microbial activity, (oxygen was not analyzed in these samples but is planned for the future). In the nutrient-amended inoculated samples, an initial decrease in gas volume (-0.27 mL g^{-1} cellulose at 45 days) was followed by an increase after 69 days to 0.86 mL g^{-1} cellulose at 200 days at a rate of 0.008 mL g^{-1} cellulose day^{-1}. This rate was calculated from linear slope

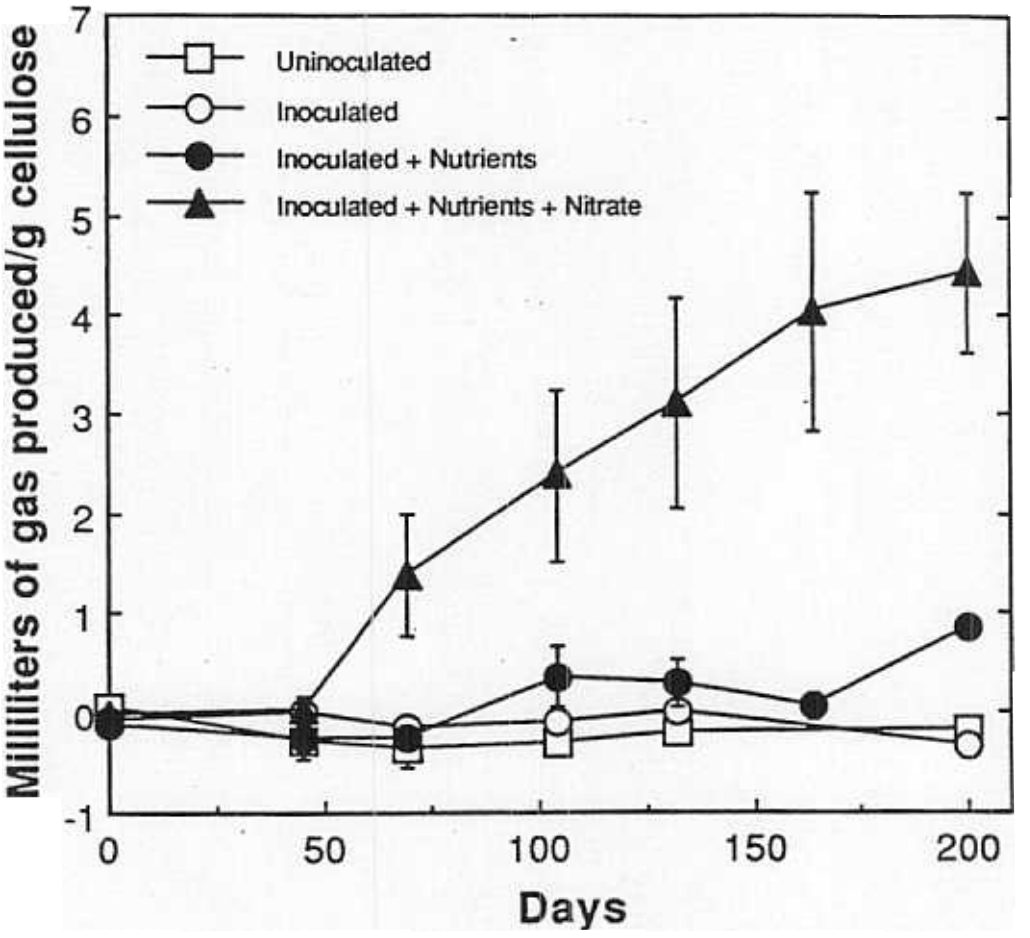

Figure 13. Total gas produced in samples incubated with an initial atmosphere of air.

of gas production from 69 to 200 days. Excess nitrate stimulated the rate of gas production (0.023 mL g^{-1} cellulose day^{-1} after 69 days) resulting in a total of 4.42 mL g^{-1} cellulose at 200 days. This stimulatory effect was also evidenced by the lack of a long lag-phase (see Figure 13) and was a result of the metabolism of dissolved carbon in the presence of nitrate.

Total gas production in aerobic samples containing bentonite is presented in Figure 14. Uninoculated and inoculated samples, with no added nutrients, did not produce gas. Inoculated samples containing nutrients produced 4.38 mL of gas g^{-1} cellulose after 200 days (Table 14, Appendix D), at a rate of 0.028 mL g^{-1} cellulose day^{-1}. In the presence of excess nitrate, the total production increased to 6.07 mL g^{-1} cellulose at 200 days at a rate of 0.034 mL g^{-1} cellulose day^{-1}. Enhanced total gas production was seen in samples containing bentonite, and was apparently due to a combination of abiotic and biotic factors, which are evident upon examining carbon dioxide evolution in the presence of bentonite.

Extrapolation of the gas production rates from mL per g cellulose per day to mol per drum of waste per year is accomplished with the following conversion factors: for an assumed average drum of transuranic waste, with about 10 Kg of cellulosic materials, a total gas generation rate of 0.01 mL g^{-1} cellulose day^{-1} corresponds to a gas generation rate of 1.6 mol gas per drum per year.

5.1.2 Carbon Dioxide Production

Uninoculated samples produced 4.00 μmol of CO_2 g^{-1} cellulose at 200 days, which was slightly less than the formalin treated controls (7.62 μmol g $cellulose^{-1}$), as shown in Figure 15 (and Table 15, Appendix D). However, inoculated samples produced 8.30 μmol of CO_2 g^{-1} cellulose, slightly higher than uninoculated and formalin-treated controls, due to the onset of microbial activity. Inoculated samples containing nutrients produced 40.8 μmol carbon dioxide g^{-1} cellulose at 200 days, at a rate of 0.283 μmol g^{-1} cellulose day^{-1}. In the presence of excess nitrate, 95.6 μmol carbon dioxide g^{-1} cellulose were produced at a rate of 0.484 μmol g^{-1} cellulose day^{-1}, more than twice that of samples without excess nitrate.

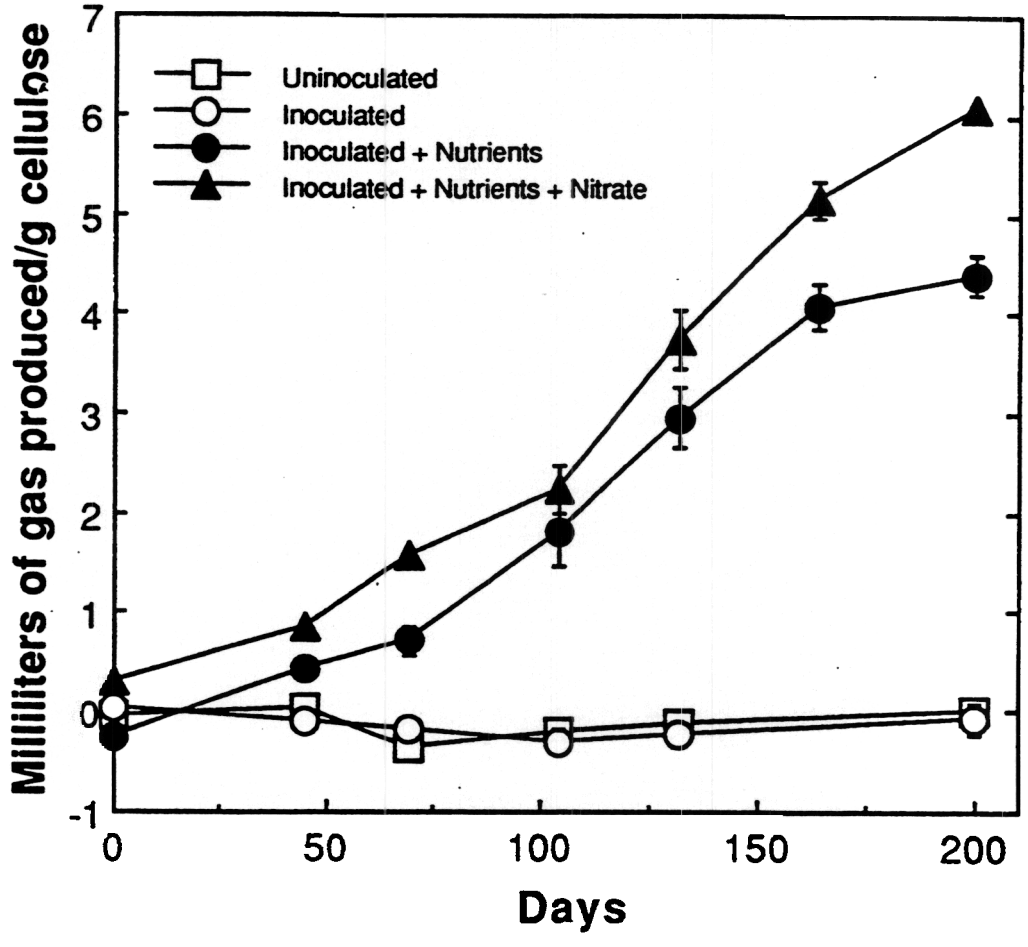

Figure 14. Total gas produced in samples containing bentonite incubated with an initial atmosphere of air.

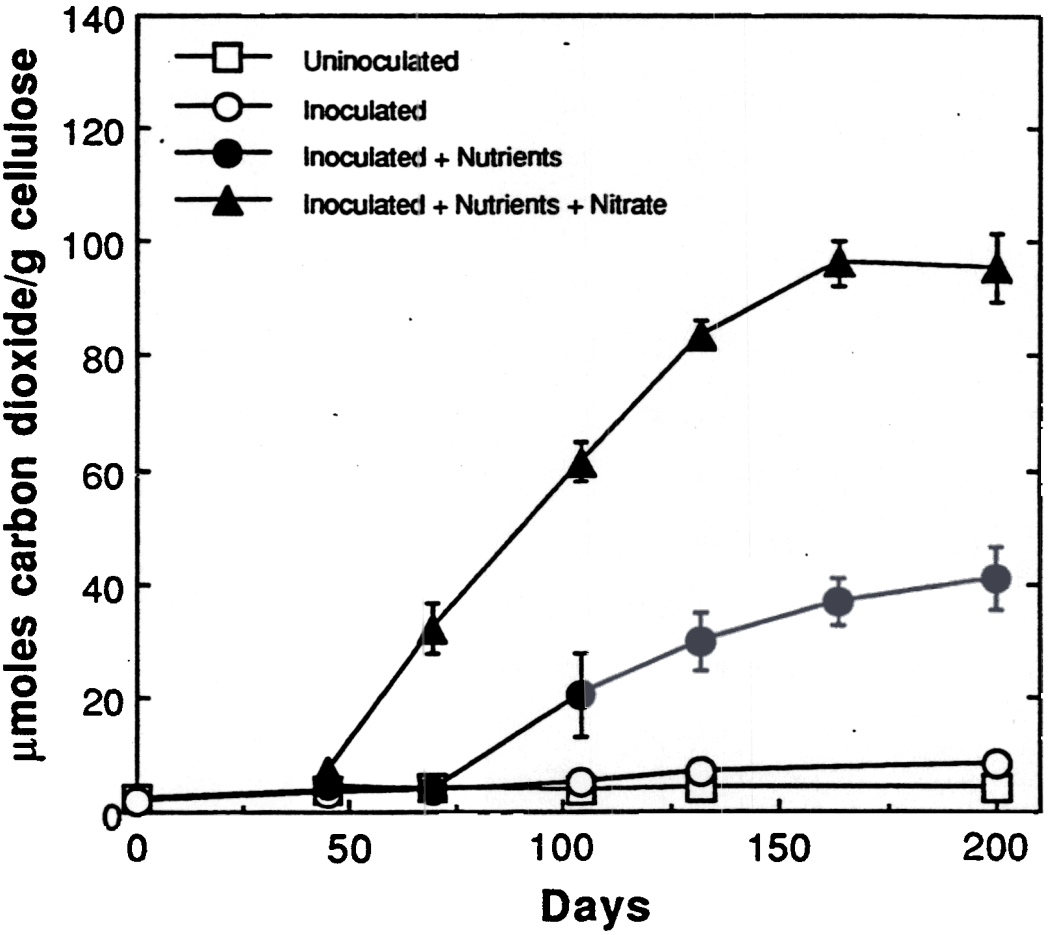

Figure 15. Carbon dioxide produced in samples incubated with an initial atmosphere of air.

Evidence of the growth of halophilic bacteria was noted in nutrient-amended samples by a red/pink color at the bottom of the bottles. This red coloration, characteristic of halophiles, is caused by the presence of bacterioruberin, a 50-carbon carotenoid pigment. This coloration was not seen in formalin-treated controls or unamended samples.

The addition of bentonite resulted in the production of a significant amount of abiotically produced carbon dioxide in samples without cellulose. Table 5, Appendix D shows that carbon dioxide increased from 17.7 μmol sample^{-1} to 40.0 μmol sample^{-1} without cellulose, inoculum and nutrients (sample 4(NC)-a), compared to the same treatment without bentonite. The latter treatment showed a slight increase, from 1.38 to 2.18 μmol sample^{-1} (see Table 2, Appendix D). Formalin-treated control samples also showed the same trend. The net effect was an increase in abiotically produced carbon dioxide (approximately 40 μmol sample^{-1}, (Table 5) by the addition of bentonite. Carbon dioxide production was insignificant in uninoculated unamended samples, (Figure 16). The inoculated unamended samples with bentonite produced 21.5 μmol g^{-1} cellulose at 200 days (Figure 16), whereas the samples without bentonite produced 8.30 μmol g^{-1} cellulose (Table 15, Appendix D). Nutrient-amended inoculated samples produced 69.8 μmol carbon dioxide g^{-1} cellulose, whereas nutrient-amended inoculated samples with excess nitrate produced 116 μmol g^{-1} cellulose at 200 days. After an initial lag of 69 days, carbon dioxide was produced at a rate of 0.533 and 0.869 μmol g^{-1} cellulose day^{-1} in nutrient-amended and nutrient-amended plus excess nitrate samples, respectively. These rates of carbon dioxide production are higher than the same treatments without bentonite (0.283 and 0.484 μmol g cellulose^{-1} day^{-1}, respectively). The buffering effect of $CaCO_3$, as well as minerals and trace elements including Fe, Al, Si and exchangeable cations (Na^+, Ca^{2+}, Mg^{2+}) and anions (SO_4^{2-}) present in the bentonite (Wanner et. al., 1992), may enhance microbial activity. Bentonite also provides an attachment site for microorganisms that may favor their growth.

Extrapolation of the carbon dioxide production rates from μmol per g^{-1} cellulose day^{-1} to mol drums^{-1} of waste year^{-1} is accomplished with the following conversion factor: for an assumed average drum of transuranic waste, with about 10 Kg of cellulosic materials, a

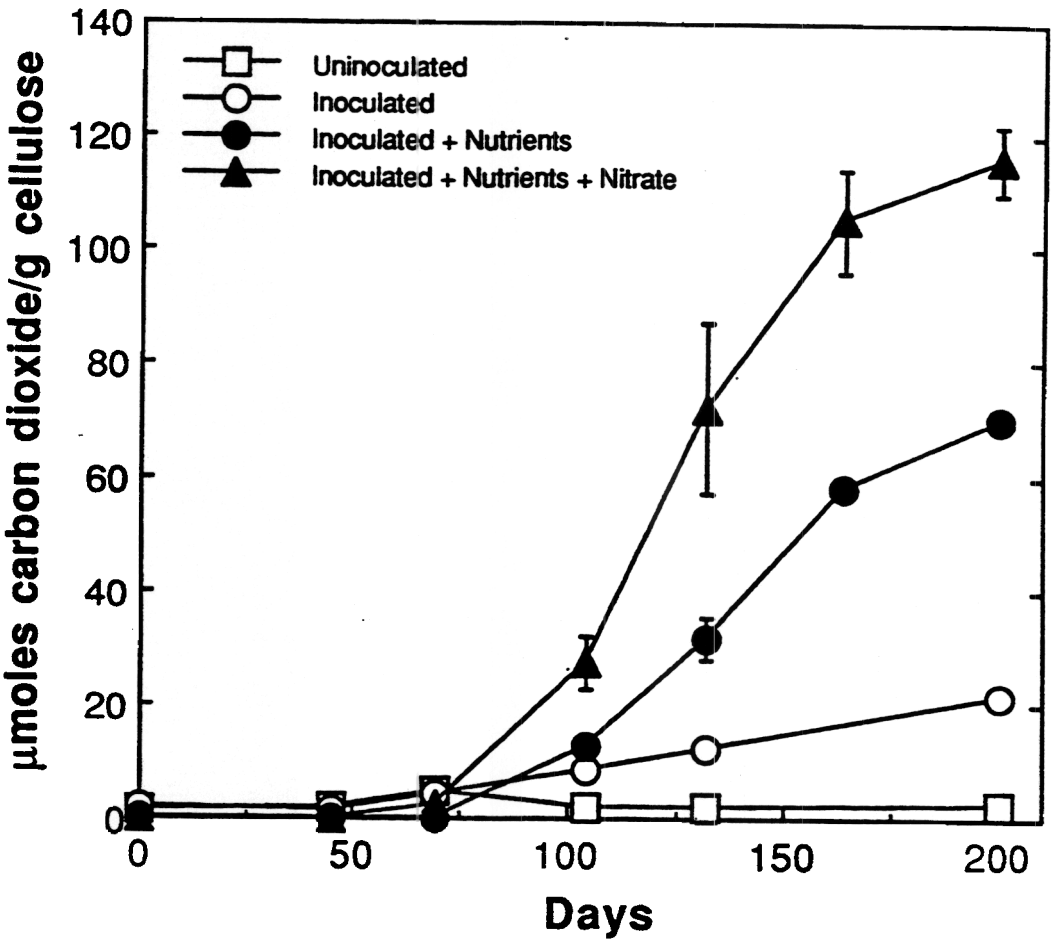

Figure 16. Carbon dioxide produced in samples containing bentonite incubated in an initial atmosphere of air.

carbon dioxide gas generation rate of 1. μmol CO_2 g^{-1} cellulose day^{-1} corresponds .7 mol of gas drum $year^{-1}$

5.1.3 Nitrous-Oxide Production

Nutrient-amended samples contained 250 μmol nitrate g^{-1} cellulose, while nutrient-amended samples plus excess nitrate contained 1240 μmol nitrate g^{-1} cellulose. Acetylene not added to samples and, therefore, the nitrous oxide is both being produced and reduced to N_2 in these samples. Nitrous oxide was not detected uninoculated inoculated samples without amendments, indicative of the lack of microbial activity see Figure 17). Nitrous oxide accumulated in the inoculated nutrient-amended samples, with production peaking 24.4 μmol g^{-1} cellulose at 132 days and then declining to 1.7 μmol g^{-1} cellulose at 64 days (Table Appendix D Nitrous-oxide produced at rate of 0.67 μmol g^{-1} cellulose day^{-1} from 69 to 104 days. In the presence of excess nitrate, nitrous oxide was produced at the rate of 0.835 μmol g^{-1} cellulose day^{-1} reaching 15 μmol g^{-1} cellulose at 200 days.

In the presence of bentonite, nitrous oxide was not detected in uninoculated and inoculated unamended samples Table 18, Appendix D and Figure 18 The addition of nutrients to inoculated samples stimulated nitrous oxide production from 69 to 104 days at 1.00 μmol g^{-1} cellulose day^{-1} Thereafter N_2O did not accumulate the headspace, probably because of depletion of available nitrate rapid conversion of N_2O to nitrogen gas. In the presence of excess nitrate, nitrous oxide was produced at rate of 0.589 μmol g^{-1} cellulose day^{-1} reaching maximum (82.7 μmol g^{-1} cellulose at 200 days. The continued accumulation of nitrous oxide at 200 days was probably due to the abundance of available nitrate. Addition of bentonite did not result in substantial accumulation of N_2O in the headspace, suggesting that N_2O was rapidly converted to N_2 as soon was formed. Samples with and without bentonite exhibited the trend (Figures 17 and 1 although less N_2O detected in the former

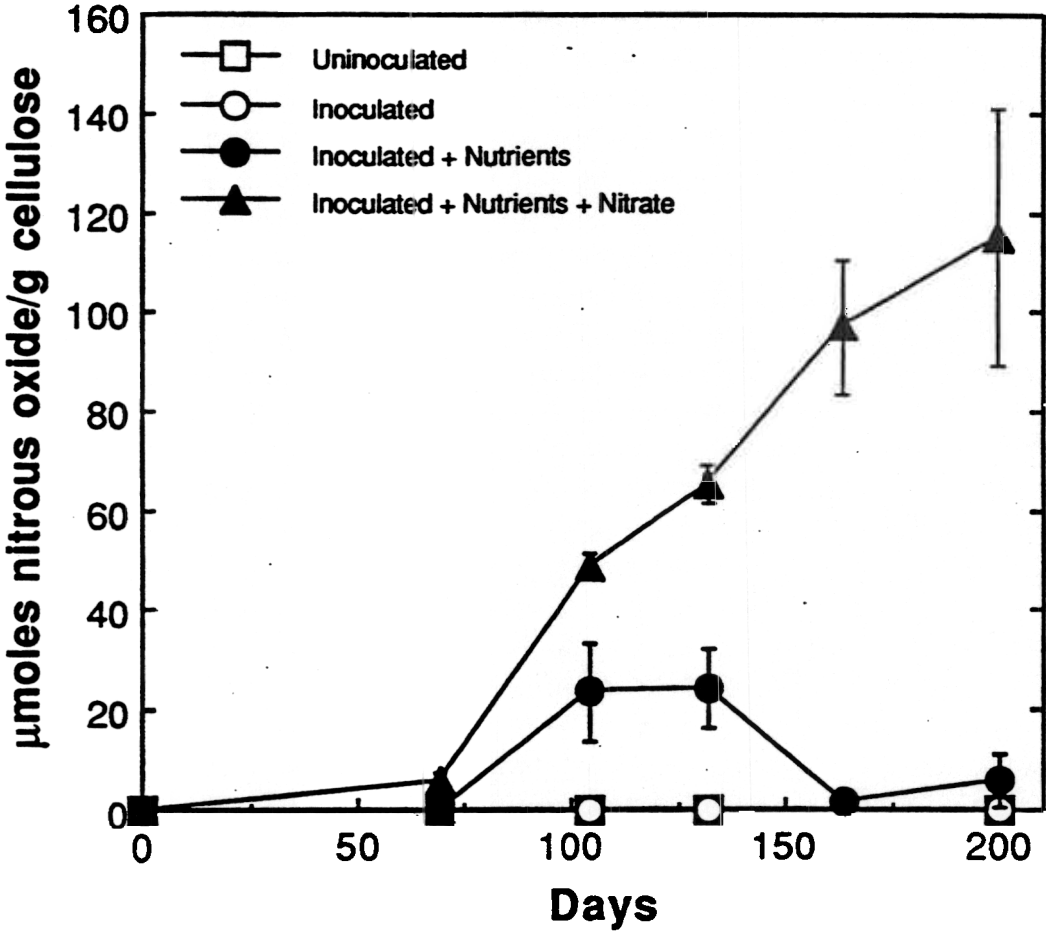

Figure 17. Nitrous oxide produced in samples incubated with an initial atmosphere of air.

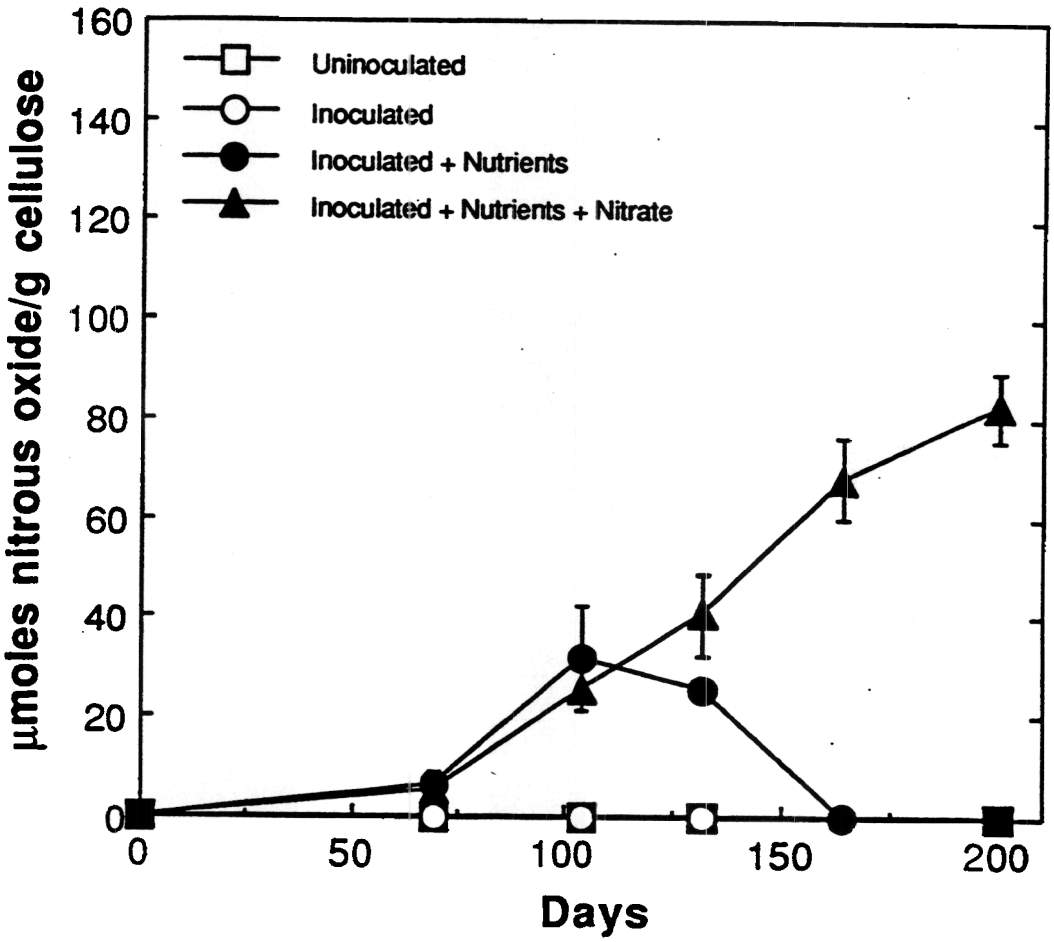

Figure 18. Nitrous oxide produced in samples containing bentonite incubated with an initial atmosphere of air.

5.2 Anaerobic Treatments

5.2.1 Total Gas Production

Figure 19 shows the total gas produced in samples incubated under anaerobic conditions in the presence of nitrogen. Uninoculated samples showed a slight loss of gas of about 3.20 mL sample^{-1}, presumably due to sampling. Inoculated samples without nutrients produced 0.59 mL g^{-1} cellulose at 200 days (see Table 19, Appendix D). After a lag of about 45 days, total gas production increased in inoculated samples containing nutrients, which produced 2.27 mL g^{-1} cellulose at a rate of 0.021 mL g^{-1} cellulose day^{-1}. With nutrients plus excess nitrate, 5.44 mL of gas were produced at a rate of 0.039 mL g^{-1} cellulose day^{-1}.

Figure 20 shows total gas produced in anaerobic samples containing bentonite. Uninoculated, unamended samples showed a net loss at 200 days to -0.28 mL g^{-1} cellulose (Table 20, Appendix D), due to a combination of sampling and data correction. Inoculated, unamended samples produced 0.81 mL g^{-1} cellulose after a lag of 69 days, at a rate of 0.007 mL g^{-1} cellulose day. In nutrient-amended samples, the addition of bentonite had no significant effect and gas production was similar to samples without bentonite (Figure 19). In contrast the addition of bentonite increased gas production in aerobic-amended samples. Inoculated amended samples produced 1.92 mL of gas at a rate of 0.013 g^{-1} cellulose day^{-1}, and inoculated samples with excess nitrate produced 3.52 mL of gas at a rate of 0.025 g^{-1} cellulose day^{-1}.

5.2.2 Carbon Dioxide Production

Uninoculated samples produced only about 3.59 μmol carbon dioxide g^{-1} cellulose over 200 days (Figure 21), but in the presence of inoculum, carbon dioxide increased to 5.47 μmol g^{-1} cellulose (Table 21, Appendix D). After a lag of 69 days, inoculated samples amended with nutrients produced 26.0 μmol carbon dioxide at a rate of 0.198 μmol g^{-1}

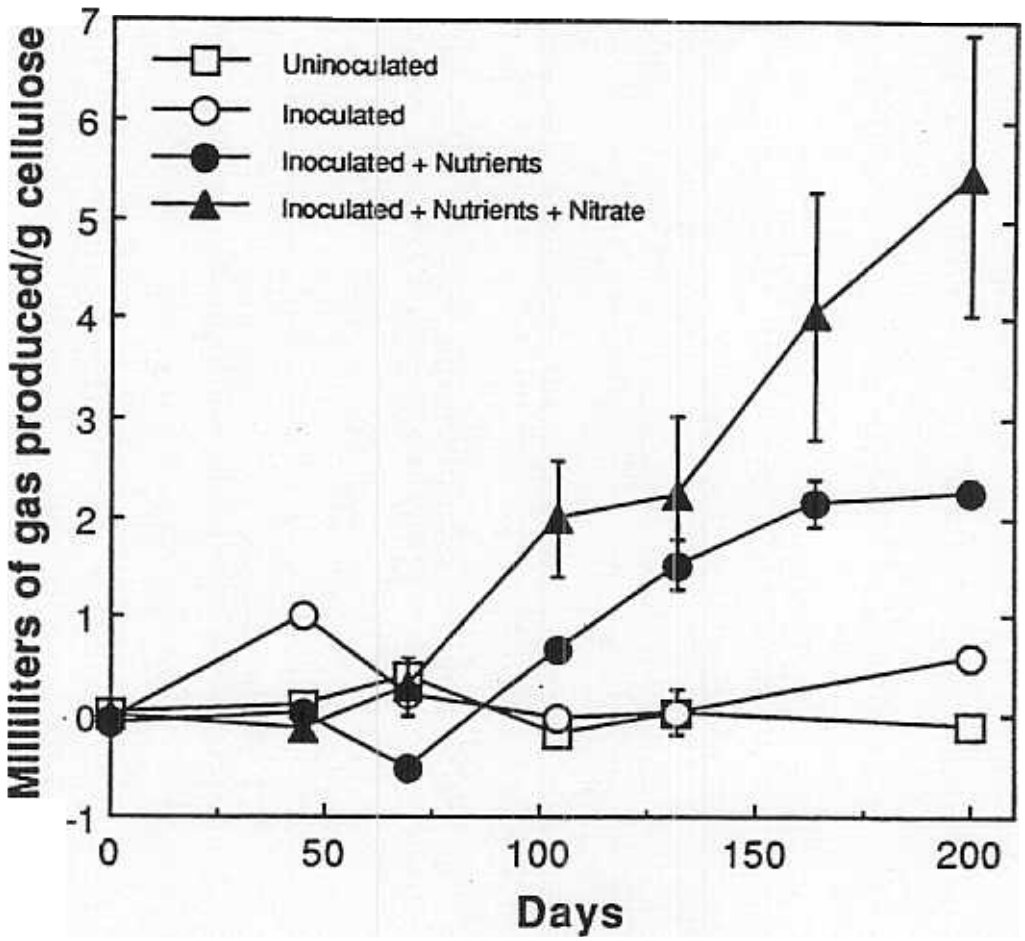

Figure 19. Total gas produced in anaerobic samples.

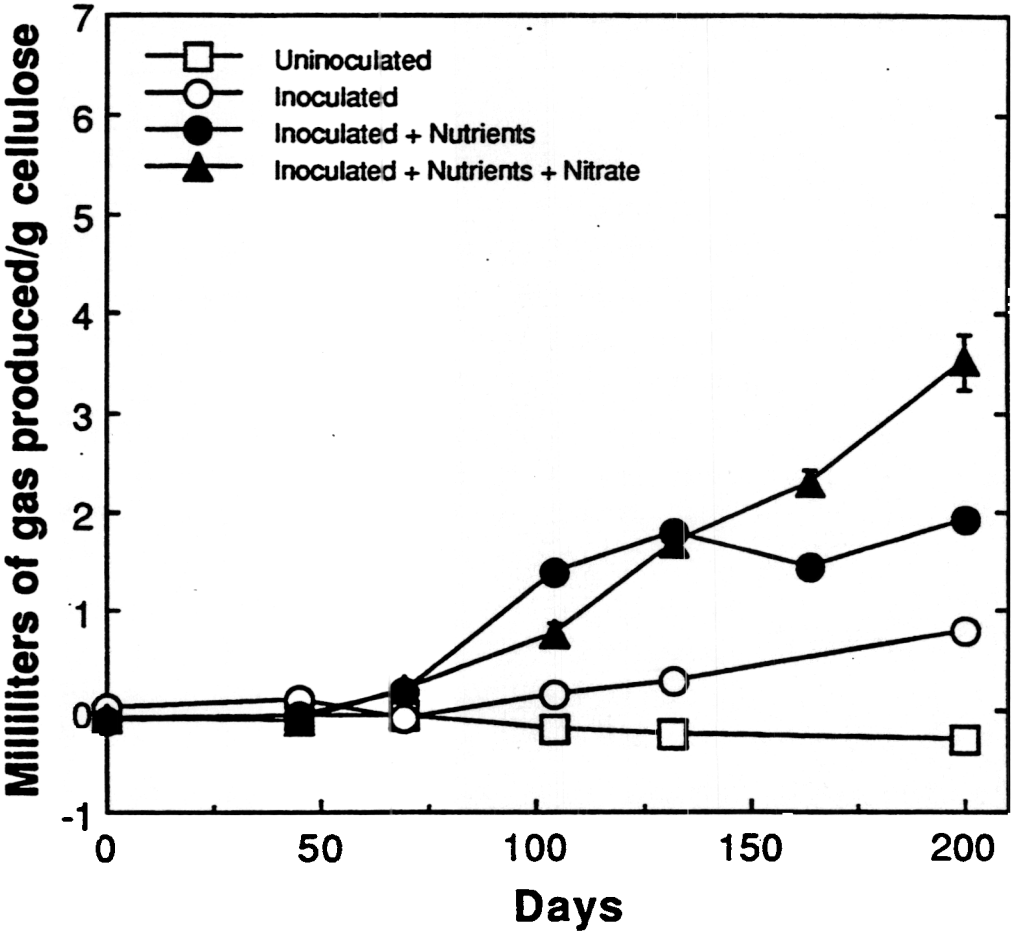

Figure 20. Total gas produced in anaerobic samples containing bentonite.

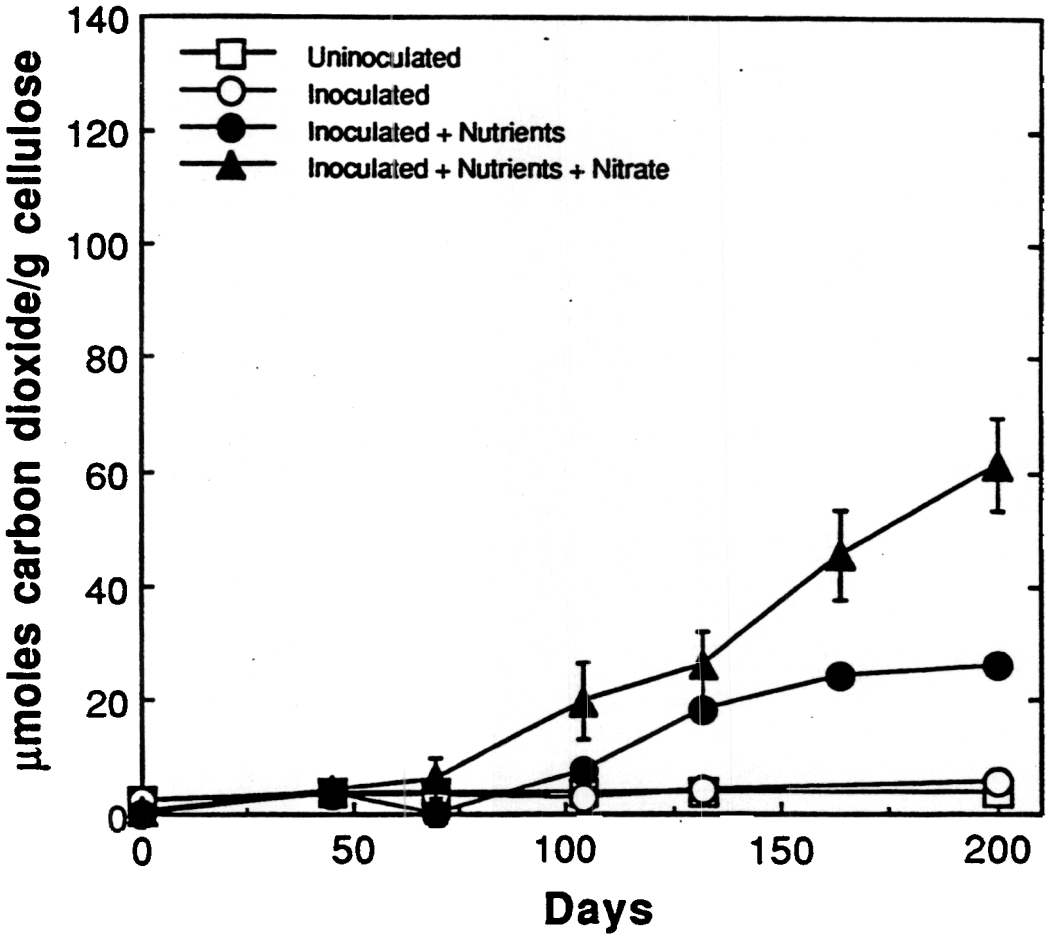

Figure 21. Carbon dioxide produced in anaerobic samples.

cellulose day^{-1} (Table 21, Appendix D). Addition of excess nitrate stimulated carbon dioxide production to 61.4 μmol at the rate of 0.422 μmol g^{-1} cellulose day^{-1}.

Figure 22 shows carbon dioxide production in anaerobic samples containing bentonite. Uninoculated unamended samples produced 0.22 μmol g^{-1} cellulose at 200 days, while inoculated unamended samples produced 8.28 μmol g^{-1} cellulose. The addition of bentonite enhanced the background (abiotic) carbon dioxide concentration by about 40.0 μmol (Table 22, Appendix D). Inoculated samples plus nutrients produced 31.8 μmol carbon dioxide g^{-1} cellulose over 200 days at a rate of 0.236 μmol g^{-1} cellulose day^{-1}. Inoculated samples with nutrients plus excess nitrate produced 35.0 μmol carbon dioxide g^{-1} cellulose over 200 days at a rate of 0.252 μmol g^{-1} cellulose day^{-1}.

5.2.3 Nitrous Oxide Production

The rate of nitrous oxide accumulation in anaerobic samples is shown in Figure 23. Nitrous oxide was not detected in either uninoculated or inoculated samples (Table 23, Appendix D). In the inoculated, amended samples, nitrous oxide accumulated to 15.5 μmol after 100 days and remained relatively unchanged up to 200 days. In the presence of excess nitrate, nitrous oxide was produced after 69 days at a rate of 0.602 μmol g^{-1} cellulose day^{-1}, reaching a concentration of 79 μmol at 200 days. In contrast, nitrous oxide accumulation was higher in aerobic samples (see Figure 15) than anaerobic samples.

Figure 24 shows nitrous oxide production in anaerobic samples containing bentonite. Nitrous oxide was not detected in uninoculated and inoculated samples (see Table 24, Appendix D). In inoculated samples with nutrients, only trace amounts of nitrous oxide were detected at 104 and 164 days. In the inoculated samples containing excess nitrate, nitrous oxide was produced at a rate of 0.647 μmol g^{-1} cellulose day^{-1} after 104 days and reached 62.1 μmol g^{-1} cellulose at 200 days. In comparison to treatments without bentonite, less nitrous oxide accumulated when bentonite was present. This may be caused by either a suppression or an enhancement of denitrification activity (enabling the complete conversion of nitrous oxide to nitrogen without accumulation of nitrous oxide).

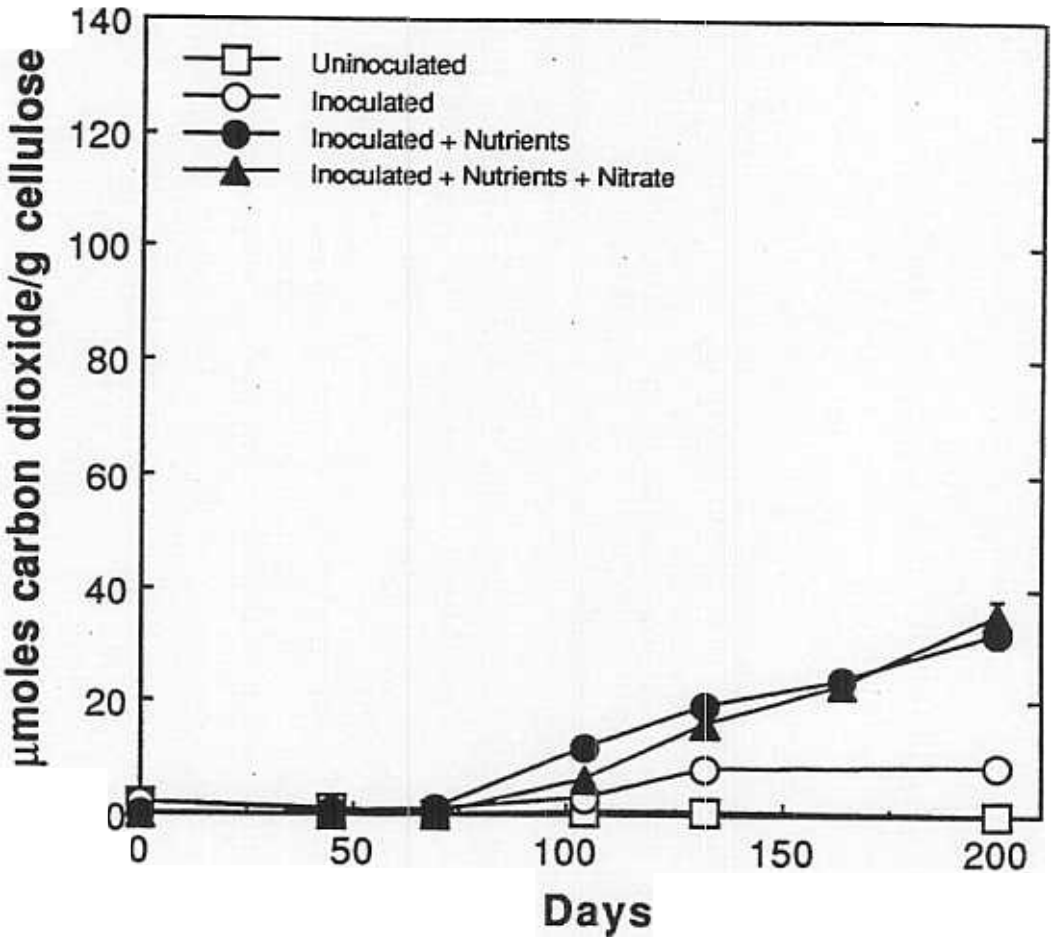

Figure 22. Carbon dioxide produced in anaerobic samples containing bentonite.

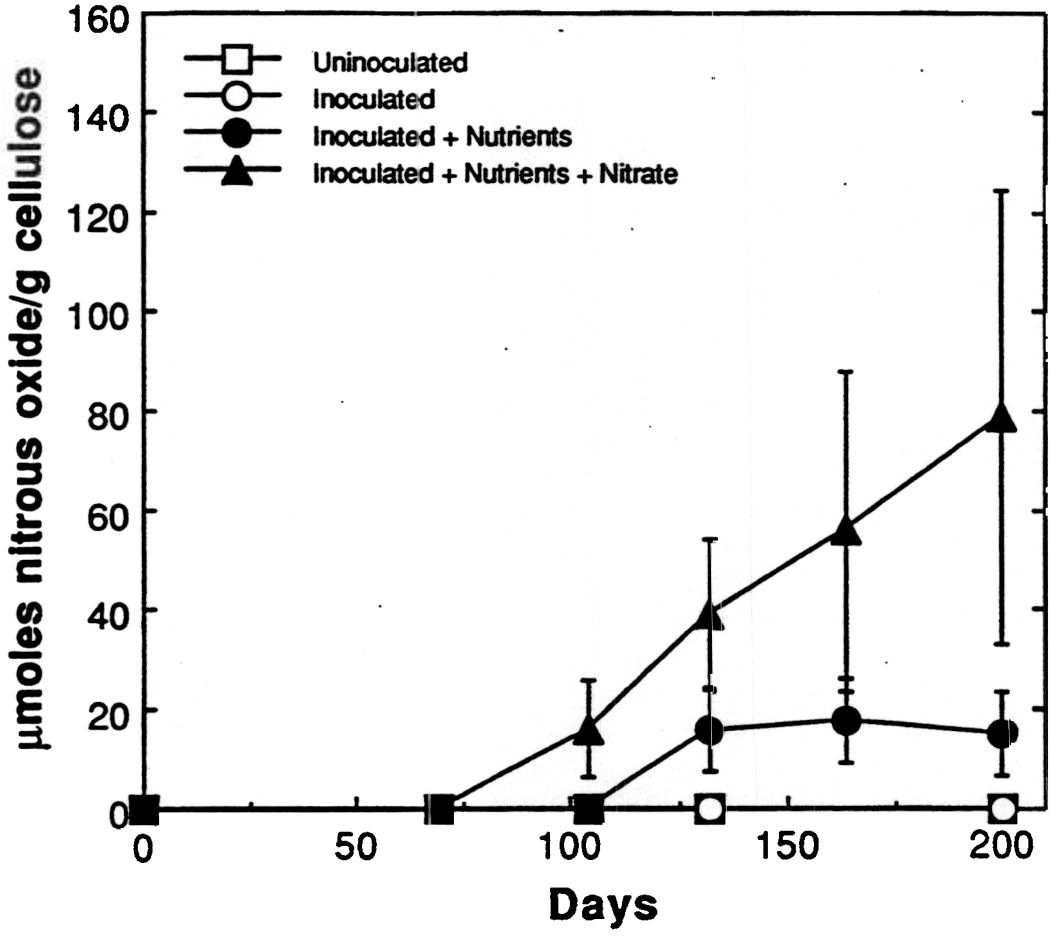

Figure 23. Nitrous oxide produced in anaerobic samples.

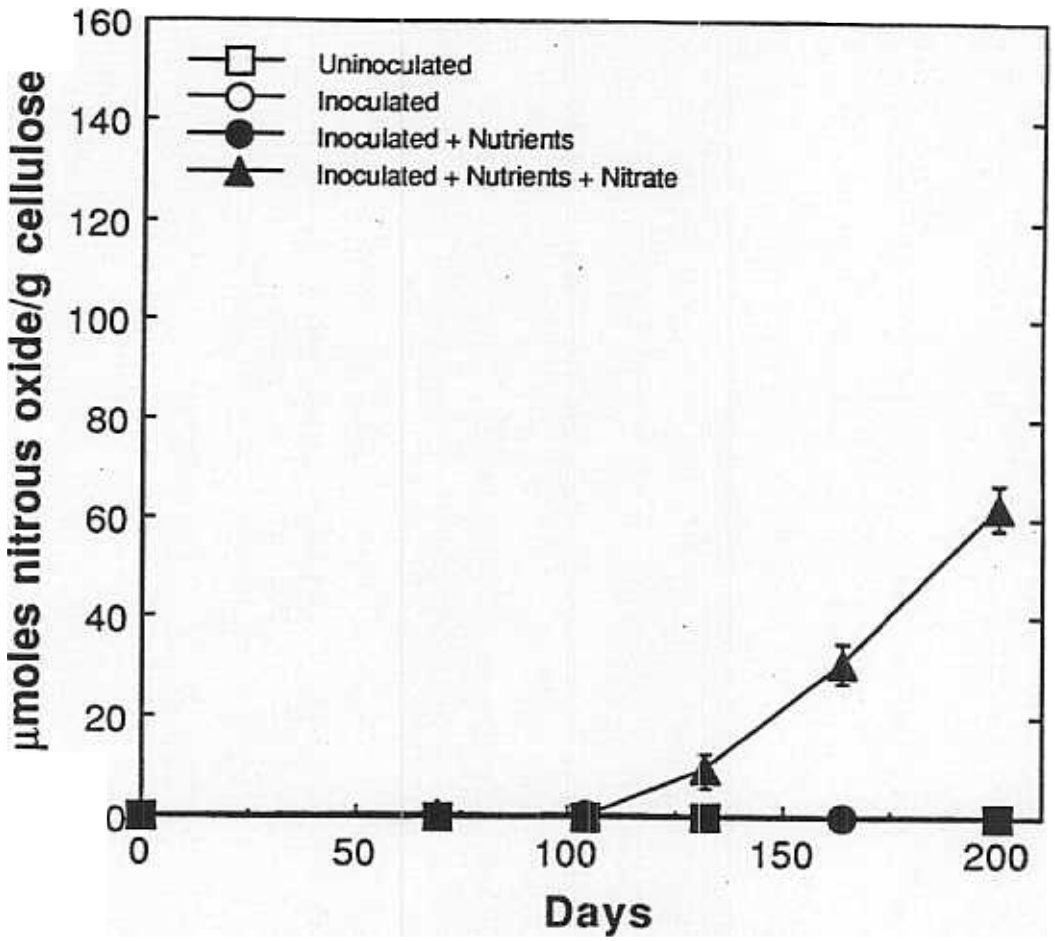

Figure 24. Nitrous oxide produced in anaerobic samples containing bentonite.

6.0 SUMMARY

Gas generation from microbial degradation of a mixture of cellulosic waste was investigated. Cellulosic waste consisting of a mixture of filter paper, paper towels, and Kimwipes® were incubated in the presence of WIPP brine with and without a mixed inoculum, nutrients, or nutrients plus excess nitrate and bentonite. Abiotic (control) samples were treated with formalin and showed no microbial activity. Nitrogen (anaerobic) or air-containing (aerobic) samples with cellulose showed an increase in total gas, CO_2, and N_2O when inoculated with a mixed inoculum without any added nutrients, with nutrients, or nutrients plus excess nitrate and bentonite. In particular, samples which received nutrients plus excess nitrate produced much more gas, CO_2, and N_2O than samples which did not. Bentonite increased the background level of CO_2 concentration due to abiotic reactions and also appears to have a stimulatory effect on aerobic microbial activity.

Table 14 summarizes the rate and extent of gas production due to the presence of cellulose from 69 to 200 days (131 days). Before 69 days, gas production in most treatments was not directly attributable to the presence of cellulose because the samples without cellulose also produced gas due to carry over of nutrients in the mixed inoculum, and metabolism of added nutrients. After 69 days, gas production in samples with cellulose exceeded those without cellulose. Gas production rates were calculated from the linear slope between 69 and 200 days. Negative values in the table denote a loss in gas volume. The negative rate reported for carbon dioxide in uninoculated treatments is the result of inactivity in these samples, and should be interpreted as "zero".

The total volume of gas produced in air-containing (aerobic) samples was highest in the presence of bentonite. Gas was produced at a rate of 0.028 mL g^{-1} cellulose day^{-1} in inoculated, nutrient-amended samples, and at 0.034 mL g^{-1} cellulose day^{-1} in inoculated, nutrient-amended samples containing excess nitrate. The highest amount of gas was produced in the presence of excess nitrate (6.07 mL g^{-1} cellulose).

Table 14. Summary of Rate and Net Gas Production in Samples Containing Cellulose

Sample	Total Volume of Gas		Carbon Dioxide		Nitrous Oxide	
	Rate* (ml/g cell./day)	Total Produced at 200 Days (ml/g cell.)	Rate* (µmol/g cell./day)	Total Produced at 200 Days (µmol/g cell.)	Rate* (µmol/g cell./day)	Total Produced at 200 Days (µmol/g cell.)
Aerobe						
Uninoculated	0.001	-0.18	-0.001	4.00	--	ND
Inoculated	-0.001	-0.34	0.033	8.30	--	ND
Inoc. + Nutrients	0.008	0.86	0.283	40.8	0.674****	24.4
Inoc. + Nut. + Nitr.	0.023***	4.42	0.484****	95.6	0.835	115
Aerobe + Bentonite						
Uninoculated	0.003	0.00	-0.016	2.32	--	0.048
Inoculated	0.001	-0.08	0.134	21.5	--	ND
Inoc. + Nutrients	0.028	4.38	0.533	69.8	1.00****	31.8****
Inoc. + Nut. + Nitr.	0.034	6.07	0.869	116	0.589	82.7
Anaerobe						
Uninoculated	-0.004	-0.09	-0.003	3.59	--	ND
Inoculated	0.003	0.59	0.016	5.47	--	ND
Inoc. + Nutrients	0.021	2.27	0.198	26.0	0.564*****	15.8*****
Inoc. + Nut. + Nitr.	0.039	5.44	0.422	61.4	0.602	79.0
Anaerobe + Bentonite						
Uninoculated	-0.003	-0.28	-0.005	0.22	--	ND
Inoculated	0.007	0.81	0.057	8.28	--	ND
Inoc. + Nutrients	0.013	1.92	0.236	31.8	--	ND
Inoc. + Nut. + Nitr.	0.025	3.52	0.252	35.0	0.647******	62.1*****

* Rate calculated from 69 days (end of lag phase) to 200 days (131 days) except where noted; rate assumed linear and averaged over available data.

**For an assumed average drum of transuranic waste, with 10 kg of cellulosic material, the following rate conversion factors are applicable:

0.01 ml/g/day = 1.6 moles/drum/year

1.0 µmole/g/day = 3.7 moles/drum/year

*** Lag phase not present, gas production started at T=0

**** Nitrous oxide reached maximum at 104 days, rate is over 35 days

***** Nitrous oxide reached maximum concentration at 132 days, rate is over 32 days.

****** Lag phase lasted 104 days

Negative values denote gas volume loss or decrease in concentration over time.

ND - not detected

In anaerobic samples, gas production was highest in the absence of bentonite. Gas was produced at a rate of 0.021 mL g^{-1} cellulose day^{-1} in inoculated, nutrient-amended samples, and 0.039 ml g^{-1} cellulose day^{-1} in inoculated, nutrient-amended samples containing excess nitrate.

The concentration of carbon dioxide was highest in aerobic treatments in the presence of bentonite. In samples containing excess nitrate, 116 μmol CO_2 g^{-1} cellulose was produced at a rate of 0.869 μmol g^{-1} cellulose day^{-1}. Inoculated, unamended samples of this treatment also showed the highest amount of carbon dioxide, with 21.5 μmol g^{-1} cellulose produced over 200 days. In the absence of bentonite, aerobic samples that were inoculated with nutrients produced 40.8 μmol CO_2 g^{-1} cellulose, whereas the samples containing excess nitrate produced 95.6 μmol CO_2 g^{-1} cellulose. Aerobic samples produced more carbon dioxide than anaerobic samples. In the absence of bentonite, anaerobic samples produced 0.422 μmol CO_2 g^{-1} cellulose day^{-1} with a total yield 61.4 μmol CO_2 g^{-1} cellulose in the presence of excess nitrate. However, in the presence of bentonite, 35.0 μmol g^{-1} cellulose was produced in anaerobic samples containing excess nitrate, about one third of that produced in aerobic samples. Therefore, initially aerobic processes were more efficient in producing CO_2.

In the presence of cellulose and nutrients, there is significantly greater amount of gas production than in samples without nutrients. In the absence of nutrients, microbial activity was minimal. Aerobic, denitrifying, and anaerobic, primarily fermentative, activities, were the predominant microbial processes noted.

Production of nitrous oxide correlated with the presence of excess nitrate (1240 μmol g^{-1} cellulose), and 115 μmol g^{-1} cellulose was produced in aerobic samples without bentonite. Nitrous oxide did not accumulate in nutrient-amended samples which contained 0.1% nitrate (250 μmol g^{-1} cellulose); it quickly disappeared after about 30 days. Bentonite did not stimulate the accumulation of nitrous-oxide, but instead, was correlated with a lower accumulation of N_2O.

The long-term inundated experiments showed enhanced halophilic bacterial activity in the presence of cellulose under aerobic and anaerobic conditions. Up to 200 days, gas production was highest in nutrient amended samples including excess nitrate treatments containing an initial concentration of oxygen; this was enhanced by the addition of bentonite.

Table 15 presents gas production data scaled up to total gas and carbon dioxide produced per drum of waste per year. Nitrous oxide is not presented because this gas, the production of which is significant as a marker of microbial activity, is converted to nitrogen which affects the production rate. The rates drum^{-1} of waste year^{-1} were calculated on the basis of an assumed average drum of transuranic waste with about 10 Kg of cellulosic material. A total gas generation rate of 0.01 mL of gas g^{-1} cellulose day^{-1} corresponds to a generation rate of 1.6 mol of gas drum^{-1} year^{-1}. A carbon dioxide generation rate of 1.0 μmol CO_2 g^{-1} cellulose day^{-1} corresponds to a generation rate of 1.6 mol gas drum^{-1} year^{-1}.

The data contained in this report is a summarization of work in progress, (a status report) and should not be interpreted as final values. Most of the long-term studies are still in progress. Gas production rates will undoubtedly be modified after long-term data, up to about two years or longer, are obtained and analyzed. The preliminary data included herein, and resultant gas production rates, should only be used for preliminary interpretations and tentative conclusions. Further data and interpretation from this microbial degradation-gas generation study will be documented in the future.

Table 15. Summary of Gas Production Rates Expressed as per g Cellulose/Day and Scaled up to per Drum/Year.

Sample	Total Volume of Gas		Carbon Dioxide	
	Rate* (ml/g cell./day)	Calculated Amount Produced per Waste Drum** (moles/drum/year)	Rate* (μmol/g cell./day)	Calculated Amount Produced per Waste Drum** (moles/drum/year)
Aerobe				
Uninoculated	0.001	0.16	-0.001	--
Inoculated	-0.001	-0.16	0.033	0.12
Inoc. + Nutrients	0.008	1.28	0.283	1.05
Inoc. + Nut. + Nitr.	0.023***	3.68	0.484***	1.79
Aerobe + Bentonite				
Uninoculated	0.003	0.48	-0.016	--
Inoculated	0.001	0.16	0.134	0.5
Inoc. + Nutrients	0.028	4.48	0.533	1.97
Inoc. + Nut. + Nitr.	0.034	5.44	0.869	3.21
Anaerobe				
Uninoculated	-0.004	-0.64	-0.003	--
Inoculated	0.003	0.48	0.016	0.06
Inoc. + Nutrients	0.021	3.36	0.198	0.73
Inoc. + Nut. + Nitr.	0.039	6.24	0.422	1.56
Anaerobe + Bentonite				
Uninoculated	-0.003	-0.48	-0.005	--
Inoculated	0.007	1.12	0.057	0.21
Inoc. + Nutrients	0.013	2.08	0.236	0.87
Inoc. + Nut. + Nitr.	0.025	4.00	0.252	0.93

* Rate calculated from 69 days (end of lag phase) to 200 days (131 days) except where noted; rate assumed linear and averaged over available data.

**For an assumed average drum of transuranic waste, with 10 kg of cellulosic material, the following rate conversion factors were used:

0.01 ml/g/day = 1.6 moles/drum/year

1.0 μmole/g/day = 3.7 moles/drum/year

*** Lag phase not present, gas production started at T=0

Negative values denote gas volume loss or decrease in concentration over time

7.0 REFERENCES

Balderston, W.L., B. Sherr, and W.J. Payne. 1976. "Blockage by Acetylene of Nitrous Oxide Reduction in *Pseudomonas perfectomarinus*," *Applied Environmental Microbiology*. Vol. 31, No.4, 504-508.

Barnhart, B.J., E.W. Campbell, E. Martinez, D.E. Caldwell, and R. Hallett. 1980. *Potential Microbial Impact on Transuranic Wastes Under Conditions Expected in the Waste Isolation Pilot Plant (WIPP), Annual Report, October 1, 1978 - September 30, 1979.* LA-8297-PR. Los Alamos National, NM: Los Alamos Scientific Laboratory.

Benoit, L., C. Cailliez, E. Petitdemange, and J. Gitton. 1992. "Isolation of Cellulolytic Mesophilic *Clostridia* from a Municipal Solid Waste Digestor," *Microbial Ecology*. Vol. 23, No.2, 117-125.

Black, J., X. Tong, and D. Grbic-Galic. 1991. *Laboratory Studies of Microbial Degradation of Transuranic (TRU) Waste, Final Progress Report January 1, 1989 - April 30, 1991.* Stanford, CA: Environmental Engineering and Science, Department of Civil Engineering, Stanford University. (Copy on file in the Sandia WIPP Central Files, Waste Management and Transportation Library, Sandia National Laboratories, Albuquerque, NM).

Bott, T.L., and L.A. Kaplan. 1991. "Selection of Surrogates for a Genetically Engineered Microorganism with Cellulolytic Capability for Ecological Studies in Streams," *Canadian Journal of Microbiology*. Vol. 37, No. 11, 848-857.

Brush, L.H. 1990. *Test Plan for Laboratory and Modeling Studies of Repository and Radionuclide Chemistry for the Waste Isolation Pilot Plant*. SAND90-0266. Albuquerque, NM: Sandia National Laboratories.

Brush, L.H. 1991. "Appendix A: Current Estimates of Gas Production Rates, Gas Production Potentials, and Expected Chemical Conditions Relevant to Radionuclide Chemistry for the Long-Term WIPP Performance Assessment," *Preliminary Comparison with 40 CFR Part 191, Subpart B for the Waste Isolation Pilot Plant, December 1991. Vol. 3: Reference Data.* WIPP Performance Assessment Division. Eds. R.P. Rechard, A.C. Peterson, J.D. Schreiber, H.J. Iuzzolino, M.S. Tierny, and J.S. Sandha. SAND91-0893/3. Albuquerque, NM: Sandia National Laboratories. A-25 through A-36.

Brush, L.H. , D. Grbic-Galic, D.T. Reed, X. Tong, R.H. Vreeland, and R.E. Westerman. 1990. "Preliminary Results of Laboratory Studies of Repository Chemistry for the Waste Isolation Pilot Plant," *Scientific Basic for Nuclear Waste Management XIV, Boston, MA, November 26-29, 1990.* Eds. T.A. Abrajano, Jr. and L.H. Johnson. SAND90-1031C. Pittsburgh, PA: Materials Research Society. Vol. 212, 893-900.

Caldwell, D.E., R.C. Hallet, M.A. Molecke, E. Martinez, and B.J. Barnhart. 1988. *Rates of CO_2 Production from the Microbial Degradation of Transuranic Wastes Under Simulated Geologic Isolation Conditions.* SAND87-7170. Albuquerque, NM: Sandia National Laboratories.

Christensen, S., and J.M. Tiedje. 1988. "Sub-Parts-Per-Billion Nitrate Method: Use of an N_2O-Producing Denitrifier to Convert NO_3- or $^{15}NO_3$- to N_2O," *Applied Environmental Microbiology.* Vol. 54, No. 6, 1409-1413.

Colberg, P.J., and L.Y. Young. 1982. "Biodegradation of Lignin-Derived Molecules Under Anaerobic Conditions," *Canadian Journal of Microbiology.* Vol. 28, No.7, 886-889.

Coleman, A.W. 1980. "Enhanced Detection of Bacteria in Natural Environments by Fluorochrome Staining of DNA," *Limnology and Oceanography.* Vol. 25, No. 5, 948-951.

DeLong, E.F., G.S. Wickham, and N.R. Pace. 1989. "Phylogenetic Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells," *Science*. Vol. 243, No.4896, 1360-1363.

Dicker, H.J., and D.W. Smith. 1985. "Effects of Organic Amendments on Sulfate Reduction Activity, H_2 Consumption, and H_2 Production in Salt Marsh Sediments," *Microbial Ecology*. Vol. 11, No. 4, 299-315.

Francis, A.J. 1985. Low-level Radioactive Wastes in Subsurface Soils," *Soil Reclamation Processes: Microbiological Analyses and Applications*. Eds. R.L. Tate III and D.A. Klein, New York, NY: Marcel Decker, Inc., 279-331.

Francis, A.J. 1990. "Microbial Dissolution and Stabilization of Toxic Metals and Radionuclides in Mixed Wastes," *Experientia*. Vol. 46, No. 8, 840-851.

Francis, A.J., S. Dobbs, and R.F. Doering. 1980a. "Biogenesis of Tritiated and Carbon-14 Methane from Low-Level Radioactive Waste," *Nuclear Chemical Waste Management*. Vol. 1, No. 1, 153-159.

Francis, A.J., S. Dobbs, and B.J. Nine. 1980b. "Microbial Activity of Trench Leachates from Shallow-Land, Low-Level Radioactive Waste Disposal Sites," *Applied and Environmental Microbiology*. Vol. 40, No. 1, 108-113.

Francis, A.J., J.M. Slater, and C.J. Dodge. 1989. "Denitrification in Deep Subsurface Sediments," *Geomicrobiology Journal*. Vol. 7, No. 1-2, 103-116.

Francis, A.J., C.J. Dodge, J.B. Gillow, and J.E. Cline. 1991. "Microbial Transformations of Uranium in Wastes," *Radiochimica Acta*. Vol. 52/53, pt. 2, 311-316.

Hope, C.F.A., and R.G. Burns. 1985. "The Barrier-Ring Plate Technique for Studying Extracellular Enzyme Diffusion and Microbial Growth in Model Soil Environments," *Journal of General Microbiology*. Vol. 131, pt. 5, 1237-1243.

Hrmová, M., E. Petráková and P.Belly. 1991. "Induction of Cellulose- and Xylan-Degrading Enzyme Systems in *Aspergillus terreus* by Homo- and Hetero-Disaccharides Composed of Glucose and Xylose," *Journal of General Microbiology*. Vol. 137, pt. 3, 541-547.

Javor, B.J. 1984. "Growth Potential of Halophilic Bacteria Isolated from Solar Salt Environments: Carbon Sources and Salt Requirements," *Applied and Environmental Microbiology*. Vol. 48, No.2, 352-360.

Kearns, J.T., C.R. Clayton, G.P. Halala, J.B. Gillow, and A.J. Francis. 1992. "Application of XPS to the Study of MIC," *Materials Performance*. Vol. 31, No. 10, 48-51.

Kushner, D.J., and M. Kamekura. 1988. "Chapter 5: Physiology of Halophilic Eubacteria," *Halophilic Bacteria*. Ed. F. Rodriquez-Valera. Boca Raton, FL: CRC Press, Inc. Vol. I, 110.

Lappin, A.R., R.L. Hunter, D.P. Garber, and P.B. Davies, eds. 1989. *Systems Analysis, Long-Term Radionuclide Transport, and Dose Assessments, Waste Isolation Pilot Plant (WIPP), Southeastern New Mexico; March 1989*. SAND89-0462, Albuquerque, NM: Sandia National Laboratories.

Laube, V.M., and S.M. Martin. 1981. "Conversion of Cellulose to Methane and Carbon Dioxide by Triculture of *Acetovibrio cellulyticus*, *Desulfovibrio sp.*, and *Methanosarcina barkeri*," *Applied and Environmental Microbiology*, Vol. 42, No. 3, 413-420.

Lovely, D.R., E.J. Phillips, Y.A. Gorby, and E.R. Landa. 1992. "Microbial Reduction of Uranium," *Nature*. Vol. 350, 413-416.

Lorowitz, W.H., D.P. Nagle, Jr., and R.S. Tanner. 1992. "Anaerobic Oxidation of Elemental Metals Coupled to Methanogenesis by *Methanobacterium thermoautotrophicum*," *Environmental Science and Technology*. Vol. 26, No. 8, 1606-1610.

Lynd, L.R., H.E. Grethlein, and R.H. Wolkin. 1989. "Fermentation of Cellulosic Substrates in Batch and Continuous Culture by *Clostridium thermocellum*," *Applied and Environmental Microbiology*. Vol. 55, No. 12, 3131-3139.

Molecke, M.A. 1979. *Gas Generation from Transuranic Waste Degradation: Data Summary and Interpretation*. SAND79-1245. Albuquerque, NM: Sandia National Laboratories.

Molecke, M.A. 1983. *A Comparison of Brines Relevant to Nuclear Waste Experimentation*. SAND83-0516. Albuquerque, NM: Sandia National Laboratories.

Ng, T.K., P.J. Weimer, and J.G. Zeikus. 1977. "Cellulolytic and Physiological Properties of *Clostridium thermocellum*," *Archives Microbiology*. Vol. 114, No. 1, 1-7.

Pavlostathis, S.G., and E. Giraldo-Gomez. 1991. "Kinetics of Anaerobic Treatment: A Critical Review," *Critical Reviews in Environmental Control*. Vol. 21, No. 5-6, 411-490.

Pavlostathis, S.G., T.L. Miller, and M.J. Wolin. 1988. "Fermentation of Insoluble Cellulose by Continuous Cultures of *Ruminococcus albus*," *Applied and Environmental Microbiology*. Vol. 54, No. 11, 2655-2659.

Postgate, J.R. 1984. *The Sulphate-Reducing Bacteria.* 2nd ed. New York, NY: Cambridge University Press. 56-100.

Poulsen, O.M., and L.W. Petersen. 1992. "Degradation of Microcrystalline Cellulose: Synergism Between Different Endoglucanases of *Cellulomonas sp.* ATCC 21399," *Biotechnology and Bioengineering.* Vol. 39, No.1, 121-123.

Priest, F.G. 1984. *Extracellular Enzymes.* Wokingham Berkshire , England: Van Nostrad Reinhold (UK) Co. Ltd. 42-43.

Rodriquez-Valera, F., ed. 1988. *Halophilic Bacteria.* Boca Raton, FL: CRC Press, Inc. Vol. I, 72.

Ross, H.N.M., M.D. Collins, B.J. Tindall, and W.D. Grant. 1981. "A Rapid Procedure for the Detection of Archaebacterial Lipids in Halophilic Bacteria," *Journal of General Microbiology.* Vol. 123, pt. 1, 75-80.

Skujins, J. 1976. "Extracellular Enzymes in Soil," *CRC Critical Reviews in Microbiology.* Vol. 4, No. 4, 383-421.

Tiedje, J.M. 1988. "Ecology of Denitrification and Dissimilatory Nitrate Reduction to Ammonium," *Biology of Anaerobic Microorganisms.* Ed. A.J.B. Zehnder, New York, NY: John Wiley and Sons. 179-186.

Tomlinson, G.A., L.L Jahnke, and L.I. Hochstein. 1986. "*Halobacterium denitrificans* sp. nov., an Extremely Halophilic Denitrifying Bacterium," *International Journal of Syst. Bacteriology.* Vol. 36, No. 1, 66-70.

Ventosa, A. 1988. "Chapter 3: Taxonomy of Moderately Halophilic Heterotrophic Eubacteria," *Halophilic Bateria.* Ed. F. Rodriquez-Valera. Boca Raton, FL: CRC Press, Inc. Vol. I, 72.

Vreeland, R., 1991. West Chester University, Pennsylania, personal communication.

Wanner H., P. Wersin, and N. Sierro. 1992. *Thermodynamic modelling of beutonite-ground interaction and implications for near field chemistry in a repository for spent fuel.* SKB Technical Report 92-37. Stockholm, Sweden: Swedish Nuclear Fuel and Waste Management, Co. 1-6.

Weimer, P.J., J.M. Lopez-Guisa, and A.D. French. 1990. "Effect of Cellulose Fine Structure on Kinetics of its Digestion by Mixed Ruminal Microorganisms in Vitro," *Applied and Environmental Microbiology*. Vol. 56, No. 8, 2421-2419.

Yoshinari, T., and R. Knowles. 1976. "Acetylene Inhibition of Nitrous Oxide Reduction by Denitrifying Bacteria," *Biochemical Biophysical Research Communications*. Vol. 69, No. 3, 705-710.

Zehnder, A.J., and W. Stumm. 1988. "Geochemistry and Biogeochemistry of Anaerobic Habitats," *Biology of Anaerobic Microorganisms*. Ed. A. J. Zehnder. New York, NY: John Wiley and Sons. 19.

Zhilina, T.N., and G.A. Zavarzin. 1990. "Extremely Halophilic Methylotrophic, Anaerobic Bacteria," *FEMS Microbiology Reviews*. Vol. 87, No. 3-4, 315-322.

APPENDIX A: DETAILS OF THE SHORT-TERM ACTIVITY MEASUREMENTS

APPENDIX A: DETAILS OF THE SHORT-TERM ACTIVITY MEASUREMENTS

The short-term experiments were developed to determine the activity of specific groups of organisms (aerobes, anaerobes, and denitrifiers) by using WIPP salt from the underground and Nash Draw brine as a basal medium and inoculum.

A concentrated stock solution of nutrients (20x) was prepared and 0.5mL dispensed into 20-mL serum bottles. Medium for anaerobes (glucose fermenters) and denitrifiers was dispensed and sealed inside a nitrogen-filled glove box. Medium for aerobes was prepared outside of the glove box in air. The samples were capped with butyl rubber stoppers and aluminum crimps and autoclaved (120°C, 20 psi, 15 minutes).

The nutrient solution was added prior to the addition of inoculum to achieve a final concentration of nutrients in the samples as follows:

Aerobe and Anaerobe Series	
glucose	5.0 g/L
yeast extract	0.5 g/L
potassium phosphate	1.0 g/L
ammonium nitrate	1.0 g/L
inoculum	9.5 mL/bottle
pH=6.8	

Denitrifier Series	
sodium succinate	5.0 g/L
yeast extract	0.5 g/L
potassium phosphate (dibasic)	1.0 g/L
ammonium nitrate	1.0 g/L
potassium nitrate	1.0 g/L
inoculum	9.5 mL/bottle
pH = 6.8	

To determine denitrification, 2 mL of acetylene was injected into the headspace and nitrous oxide production was determined by gas chromatography.

Cellulose degradation was investigated by replacing the carbon source with 0.5 g Whatman #1 filter paper.

A large volume of inoculum was prepared by dissolving 200 g of S2180, W30 muck pile salt from the WIPP underground workings into 1 L of sterile water. A 450 mL aliquot was poured into a sterile beaker and 10 mL of Laguna Cinco mud slurry and 50 mL of Laguna Cinco brine from Nash Draw were added. This was done inside the anaerobic glove box. A total of 510 mL of inoculum was prepared; 9.5 mL of this inoculum was added to each bottle of sterile nutrient medium through a sterile needle and syringe to attain a final volume of 10 mL in each bottle. The pressure was equalized after the addition of inoculum with a sterile syringe and 0.22 μm filter.

A total of 6 samples were prepared per series. Two of the 6 were treated with 1 mL of 10 % formalin to serve as a control. Six samples were also prepared without nutrient additions, 2 treated with formalin.

Samples were incubated at 30°C and analyzed for gas production (total gas, carbon dioxide and nitrous oxide) at specific time periods.

APPENDIX B: GAS ANALYSIS

APPENDIX B: GAS ANALYSIS

Total Gas

Headspace pressure was measured with a Wallace & Tiernan® digital pressure model 661-D/A035 gauge calibrated to National Institute of Standards and Technology (NIST) standard.

Carbon Dioxide

Carbon dioxide analysis was performed on a gas chromatograph equipped with a thermal conductivity detector. Instrument conditions are listed below:

Column	SS 12' x 1/8" Porapak QS
Column temp (°C)	100
Carrier gas	He
Carrier flow (mL/min)	35
Injector temp (°C)	150
Manifold temp (°C)	210
Detector temp (°C)	250
Detector current (mA)	225
Detection limit (nmol/mL)	1

Instrument calibrated with gas standards traceable to NIST.

Nitrous Oxide

Nitrous oxide analysis was performed on a gas chromatograph equipped with an electron capture detector (^{63}Ni). Instrument conditions are listed below:

Column	SS 12' x 1/8" Porapak QS
Column temp (°C)	70
Carrier gas	N_2
Carrier flow (mL/min)	30
Injector temp (°C)	270
Detector temp (°C)	270
Detector current (nA)	2
Detection limit (pmol/mL)	100

Instrument calibrated with gas standards traceable to NIST.

APPENDIX C: DETAILS OF THE LONG-TERM EXPERIMENT

APPENDIX C: DETAILS OF THE LONG-TERM EXPERIMENT

The following is a detailed description of the samples prepared for the long-term inundated experiment, including the number and chemical composition of treatments.

Sample Preparation

Detergent (Alconox®) and acid-washed (10% HCl) 160 mL serum bottles were rinsed with deionized water. They were then dried in a drying oven, covered with aluminum foil and autoclaved, thus completing preparation of the bottles.

Four paper types were used for the experiment:

Whatman #1 filter paper
Brown paper towel
White paper towel
Kimwipes

The papers were reduced to approximately 1 cm by 1 cm squares.

The bottles were filled with the processed paper. The papers were mixed together prior to filling and 1.25 g of each paper type were added to each bottle for a total of 5 grams of paper per bottle.

Anaerobic Samples

Ten liters of G-Seep #9 were removed from storage at 4°C and equilibrated overnight to room temperature. Storage at 4°C was necessary in order to prevent microbial activity in the storage containers; which could possibly pre-enrich the brine with specific microbes.

Sixty filled bottles (w/ paper) were arranged and prepared for treatment as follows:

<u>36 w/paper: unamended (G-Seep w/o additions)</u>
18 w/o bentonite
18 w/ bentonite

<u>12 w/paper: amended (G-Seep w/ nutrients)</u>
6 w/o bentonite
6 w/ bentonite

12 w/paper: amended w/ excess nitrate (G-Seep w/ nutrients and excess nitrate)
6 w/o bentonite
6 w/ bentonite

Thirty-two bottles w/o paper were arranged and prepared for treatment as follows:

16 unamended (G-Seep w/o additions)
8 w/o bentonite
8 w/ bentonite

8 amended (G-Seep w/ nutrients)
4 w/o bentonite
4 w/ bentonite

8 amended w/ excess nitrate (G-Seep w/ nutrients and excess nitrate)
4 w/o bentonite
4 w/ bentonite

A total of 92 bottles were prepared for anaerobic treatment.

Aerobic Samples

Sixty filled bottles (w/ paper) were arranged and prepared for treatment as follows:

36 w/paper: unamended (G-Seep w/o additions)
18 w/o bentonite
18 w/ bentonite

12 w/paper: amended (G-Seep w/ nutrients)
6 w/o bentonite
6 w/ bentonite

12 w/paper: amended w/ excess nitrate (G-Seep w/ nutrients and excess nitrate)
6 w/o bentonite
6 w/ bentonite

Thirty-two bottles w/o paper were arranged and prepared for treatment as follows:

16 unamended (G-Seep w/o additions)
8 w/o bentonite
8 w/ bentonite

8 amended (G-Seep w/ nutrients)
4 w/o bentonite
4 w/ bentonite

8 amended w/ excess nitrate (G-Seep w/ nutrients and excess nitrate)
4 w/o bentonite
4 w/ bentonite

A total of 92 bottles were prepared for aerobic treatment.

Nutrient Additions

The following lists the quantities added and final concentrations of nutrients in the samples. This list applies to both the aerobic and anaerobic samples with and without paper.

(a) Amended: The following quantities of nutrients were used for the amended treatments:

	grams/L	mM
Ammonium nitrate	1	12.5
Potassium phosphate (dibasic)	1	
Yeast extract (0.05%)	0.5	

Each 100 mL sample contained the following final concentration of nutrients:

	grams/100mL	µmoles
Ammonium nitrate	0.	
Potassium phosphate (dibasic)	0.1	735
Yeast extract (0.05%)	0.05	--

(b) Amended and excess nitrate added: Potassium nitrate was added in addition to the ammonium nitrate:

	grams/L	mM
Ammonium nitrate	1	12.5
Potassium phosphate (dibasic)	1	7.35
Potassium nitrate	5	49.5
Yeast extract (0.05%)	0.5	--

Each 100 mL sample contained the following final concentration of nutrients:

	grams/100mL	µmoles
Ammonium nitrate	0.1	1250
Potassium phosphate (dibasic)	0.1	735
Potassium nitrate	0.5	4950
Yeast extract (0.05%)	0.05	--

(c) Glucose added (instead of paper): Samples were prepared without paper with a glucose addition to determine the ability of the inoculum to grow in the amended samples. The treatment was composed of the following:

	grams/L	
Glucose	5	27.7
Ammonium nitrate	1	12.5
Potassium phospate (dibasic)		7.35
Yeast extract (0.05%)	0.5	--

Each 100 mL sample contains the following quantity of nutrients:

	grams/100mL	µmoles
Glucose	0.5	2770
Ammonium nitrate	0.1	1250
Potassium phosphate (dibasic)	0.1	735
Yeast extract (0.05%)	0.05	

(d) Glucose added (instead of paper) and excess nitrate: Samples were prepared without paper with a glucose addition and additional nitrate. The treatment was composed of the following:

	grams/L	
Glucose	5	27.7
Ammonium nitrate	1	12.5
Potassium phospate (dibasic)	1	7.35
Potassium nitrate	5	49.5
Yeast extract (0.05%)	0.5	

Each 100 mL sample contained the following final concentration of nutrients:

	grams/100mL	µmoles
Glucose	0.5	2770
Ammonium nitrate	0.1	1250
Potassium phosphate (dibasic)	0.1	735
Potassium nitrate	0.5	4950
Yeast extract (0.05%)	0.05	--

Sample Volumes

The final sample volume (displacement of liquid plus cellulose or liquid w/o cellulose) and the headspace volume of each treatment is as follows:

Cellulose Treatments	sample vol. (mL	hdsp. vol (mL*
U (uninoculated)	110	50
I (inoculated)	114	46
UC (uninoculated control)	113	47
IC (inoculated control)	117	43
"No paper" Treatments	**sample vol. (mL**	**hdsp. vol (mL***
NU (uninoculated)	100	60
NI (inoculated)	104	56
NUC (uninoculated control)	103	57
NIC (inoculated control)	107	53

*Headspace vol. (hdsp. vol) calculated by subtracting sample volume from volume of bottle (160ml).

Acetylene was not injected into the headspace of any of the samples in order to prevent pertubation of the headspace gas.

APPENDIX D: GAS PRODUCTION DATA (GROSS AND NET) FOR THE LONG-TERM INUNDATED EXPERIMENT

APPENDIX D: GAS PRODUCTION DATA (GROSS AND NET) FOR THE LONG-TERM INUNDATED EXPERIMENT

i. Data Reduction

Total gas, carbon dioxide, and nitrous oxide produced by aerobic and anaerobic samples up to 200 days incubation is present in Appendix D, Tables 1-12, on a per sample basis. This data was used to prepare Tables 13-24 in which total gas, carbon dioxide, and nitrous oxide production in aerobic and anaerobic treatments is presented on a per gram cellulose basis. Figures 13-24 are based on the data from Tables 13-24. The data in Tables 13-24 have been corrected for gas production in the absence of cellulose by subtracting the measured gas values in respective treatments prepared without cellulose. As an example, total gas production in aerobic samples was corrected (Table 13) by subtracting total gas production in treatments without cellulose (designated "NC", Appendix D, Table 1) from samples with cellulose (designated "C") at each period. The resultant number was divided by the total amount of cellulose in each sample bottle (5 grams) to arrive at a value that represents the total gas produced per gram of cellulose. Tables 13 to 24 represent the gas produced strictly due to the presence of cellulose in the samples, and are corrected for gas produced due to metabolism of dissolved organic carbon present in the brine or in the nutrient addition as measured in specific control treatments. Carbon dioxide was produced in certain samples in the absence of cellulose, specifically, before 69 days. After this period, gas production reported is that which is above and beyond the control treatments. Significant quantities of NO_2 were not detected in the absence of cellulose. Gas produced at time 0 was not subtracted from later values, as many seem neccessary to normalize the starting values at 0. This was not done because time 0 measurements were taken 3 days after sample preparation; therefore gas present at time 0 is due to evolution of dissolved gases and headspace equilibration. These processes contribute to the overall gas production.

Figures 13 to 24 present data from Table 13 to 24. All data in these figures are presented as gas produced per gram cellulose, and have been corrected for gas production

in the absence of cellulose. The data plotted are the mean values of three samples, and error bars represent the standard error of the mean. In the legend: "uninoculated" refers to samples that have received no mixed inoculum or nutrients; "inoculated" refers to samples that have received mixed inoculum but no nutrients; "inoculated + nutrients" refers to samples that have received both mixed inoculum and nutrients (amended), and; "inoculated + nutrients + nitrate" refers to samples that have received (i) mixed inoculum, (ii) nutrients, and (iii) excess nitrate in the form of potassium nitrate (amended plus excess nitrate).

Data for treatments prepared with glucose to determine activity of the mixed inoculum are persented in Figures 25-30. Data for gas analysis of these samples, along with formalin-treated controls for all cellulose and no-cellulose treatments, are presented in Appendix D, Tables 1(a) to 4(c).

Analysis of the samples will continue at selected intervals (as determined by gas-producing activity) past 200 days and up to 800 days. The analyses then will stop if activity ceases, as indicated by cessation of gas production, or be extended if gas-producing activity continues in the samples. If this occurs in select samples only, then only these samples will be reserved for long-term monitoring, and analyses of inactive samples will stop.

Table 1. Gross Data for the Long-Term Inundated Experiment: Total Volume of Gas Produced in Aerobic Samples.

Treatments* [Brine]	Sample Designation	Volume of Gas Produced (ml) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (1)								
Formalin treated, w/o cellulose	1(NC)-f	4.22 ± 0.13	-0.35 ± 0.13	-0.06 ± 0.45	-0.26 ± 0.06	-0.13 ± 0.13	NA	-1.44 ± 0.00
Formalin treated, with cellulose	1(C)-f	5.44 ± 0.32	4.09 ± 0.16	4.19 ± 0.10	2.08 ± 1.06	1.37 ± 0.93	NA	0.67 ± 0.90
As is, w/o cellulose	1(NC)-a	4.39 ± 0.03	3.71 ± 0.03	3.84 ± 0.03	1.90 ± 0.61	1.33 ± 0.85	NA	-0.61 ± 0.75
As is, with cellulose	1(C)-a	4.63 ± 0.10	2.28 ± 0.95	2.04 ± 0.92	0.34 ± 0.68	0.34 ± 0.61	NA	-1.53 ± 0.51
Unamended/Inoculated (2)								
Formalin treated, w/o cellulose	2(NC)-f	4.96 ± 0.02	2.88 ± 0.04	3.50 ± 0.00	2.49 ± 0.04	2.45 ± 0.14	NA	0.07 ± 0.25
Formalin treated, with cellulose	2(C)-f	4.89 ± 0.06	2.72 ± 0.38	1.76 ± 0.56	0.91 ± 0.41	0.41 ± 0.53	NA	-0.50 ± 0.44
As is, w/o cellulose	2(NC)-a	3.56 ± 0.02	2.90 ± 0.15	2.21 ± 0.30	0.53 ± 0.11	-0.91 ± 0.04	NA	-2.86 ± 0.04
As is, with cellulose	2(C)-a	3.16 ± 0.03	2.97 ± 0.22	1.44 ± 0.16	-0.44 ± 0.22	-1.28 ± 0.25	NA	-4.57 ± 0.34
Amended/Inoculated (3)								
As is, w/o cellulose	3(NC)-a	3.20 ± 0.04	1.37 ± 0.04	0.30 ± 0.00	0.11 ± 0.04	-1.49 ± 0.15	-2.48 ± 0.38	-4.53 ± 0.46
As is, with cellulose	3(C)-a	2.60 ± 0.16	0.03 ± 0.69	-0.94 ± 0.31	1.91 ± 1.53	1.44 ± 1.19	0.34 ± 0.63	-0.22 ± 0.41
As is, with glucose	3(G)-a	3.73 ± 0.08	2.29 ± 0.15	1.83 ± 0.38	-1.79 ± 0.34	-1.22 ± 1.37	1.87 ± 2.63	-1.83 ± 1.30
Excess nitrate, w/o cellulose	3(NC)-x	3.16 ± 0.08	0.84 ± 0.46	-1.30 ± 0.08	-0.78 ± 0.30	-1.14 ± 0.04	-1.80 ± 0.18	-2.82 ± 0.27
Excess nitrate, with cellulose	3(C)-x	3.04 ± 0.06	0.94 ± 0.72	5.66 ± 3.04	11.9 ± 4.4	15.5 ± 5.3	18.3 ± 6.1	19.3 ± 4.0
Excess nitrate, with glucose	3(G)-x	3.52 ± 0.02	2.74 ± 0.50	0.88 ± 0.76	-1.49 ± 0.27	-1.30 ± 0.04	-0.86 ± 0.72	2.90 ± 2.02

NA = not analyzed

Table 2. Gross Data for the Long-Term Inundated Experiment: Production of Carbon Dioxide* in Aerobic Samples

Treatments [Brine]	Sample Designation	Carbon Dioxide (μmoles/sample) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (1)								
Formalin treated, w/o cellulose	1(NC)-f	2.59 ± 0.01	2.50 ± 0.01	3.40 ± 0.40	3.32 ± 0.00	3.61 ± 0.00	NA	3.68 ± 0.01
Formalin treated, with cellulose	1(C)-f	30.5 ± 2.7	40.8 ± 0.1	44.4 ± 0.2	40.6 ± 0.8	41.8 ± 0.3	NA	41.8 ± 0.5
As is, w/o cellulose	1(NC)-a	1.38 ± 0.01	1.49 ± 0.01	0.94 ± 0.00	1.66 ± 0.01	1.83 ± 0.06	NA	2.18 ± 0.04
As is, with cellulose	1(C)-a	13.8 ± 0.5	21.1 ± 0.3	22.0 ± 0.1	21.3 ± 0.1	23.1 ± 0.1	NA	22.2 ± 0.1
Unamended/Inoculated (2)								
Formalin treated, w/o cellulose	2(NC)-f	4.97 ± 0.45	4.38 ± 0.29	4.70 ± 0.11	4.88 ± 0.3	5.02 ± 0.30	NA	4.94 ± 0.27
Formalin treated, with cellulose	2(C)-f	34.5 ± 0.8	35.6 ± 0.0	38.7 ± 0.7	34.3 ± 0.6	35.2 ± 0.1	NA	33.2 ± 1.1
As is, w/o cellulose	2(NC)-a	2.30 ± 0.02	2.97 ± 0.03	2.92 ± 0.09	5.88 ± 0.08	6.56 ± 0.11	NA	7.01 ± 0.11
As is, with cellulose	2(C)-a	12.1 ± 0.3	19.7 ± 0.8	22.6 ± 0.9	30.8 ± 0.9	40.9 ± 1.0	NA	48.5 ± 1.4
Amended/Inoculated (3)								
As is, w/o cellulose	3(NC)-a	2.87 ± 0.03	27.8 ± 1.2	72.9 ± 2.6	113 ± 5	130 ± 2	150 ± 1	142 ± 1
As is, with cellulose	3(C)-a	2.80 ± 0.10	50.9 ± 1.4	91.8 ± 5.9	215 ± 37	278 ± 25	333 ± 21	346 ± 27
As is, with glucose	3(G)-a	2.41 ± 0.03	4.03 ± 0.13	30.8 ± 2.1	94.9 ± 7.5	167 ± 32	325 ± 108	323 ± 95
Excess nitrate, w/o cellulose	3(NC)-x	2.79 ± 0.03	17.2 ± 1.9	48.9 ± 0.2	92.2 ± 0.59	115 ± 3	131 ± 2	126 ± 2
Excess nitrate, with cellulose	3(C)-x	2.60 ± 0.10	51.6 ± 0.0	210 ± 21	399 ± 18	533 ± 13	612 ± 20	604 ± 30
Excess nitrate, with glucose	3(G)-x	2.51 ± 0.01	4.05 ± 0.25	24.1 ± 5.9	72.0 ± 7.7	121 ± 4	211 ± 6	296 ± 20

*Dissolved carbon dioxide concentrations not included.

NA = not analyzed

Table 3. Gross Data for the Long-Term Inundated Experiment: Production of Nitrous Oxide* in Aerobic Samples

Treatments [Brine]	Sample Designation	Nitrous Oxide (µmoles/sample) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (1)							
Formalin treated, w/o cellulose	1(NC)-f	0.000 ± 0.000	0.003 ± 0.002	0.000 ± 0.000	0.000 ± 0.000	NA	0.001 ± 0.001
Formalin treated, with cellulose	1(C)-f	0.000 ± 0.000	0.003 ± 0.001	0.003 ± 0.001	0.013 ± 0.001	NA	0.005 ± 0.002
As is, w/o cellulose	1(NC)-a	0.000 ± 0.000	0.162 ± 0.110	0.000 ± 0.000	0.000 ± 0.000	NA	0.051 ± 0.035
As is, with cellulose	1(C)-a	0.000 ± 0.000	0.003 ± 0.001	0.006 ± 0.001	0.015 ± 0.001	NA	0.008 ± 0.000
Unamended/Inoculated (2)							
Formalin treated, w/o cellulose	2(NC)-f	0.000 ± 0.000	0.025 ± 0.018	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
Formalin treated, with cellulose	2(C)-f	0.000 ± 0.000	0.001 ± 0.000	0.002 ± 0.000	0.014 ± 0.002	NA	0.004 ± 0.000
As is, w/o cellulose	2(NC)-a	0.003 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.016 ± 0.011	NA	0.062 ± 0.007
As is, with cellulose	2(C)-a	0.014 ± 0.000	0.002 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	NA	0.009 ± 0.006
Amended/Inoculated (3)							
As is, w/o cellulose	3(NC)-a	0.000 ± 0.000	0.061 ± 0.043	0.087 ± 0.061	0.024 ± 0.003	0.000 ± 0.000	0.050 ± 0.033
As is, with cellulose	3(C)-a	0.004 ± 0.000	0.182 ± 0.147	118 ± 48	122 ± 39	8.81 ± 5.98	29.2 ± 25.2
As is, with glucose	3(G)-a	0.000 ± 0.000	0.056 ± 0.010	0.252 ± 0.122	5.64 ± 3.40	114 ± 80	105 ± 74
Excess nitrate, w/o cellulose	3(NC)-x	0.000 ± 0.000	0.001 ± 0.000	0.004 ± 0.002	0.006 ± 0.004	0.006 ± 0.001	0.023 ± 0.006
Excess nitrate, with cellulose	3(C)-x	0.000 ± 0.000	28.2 ± 8.8	245 ± 11	326 ± 18	484 ± 67	577 ± 129
Excess nitrate, with glucose	3(G)-x	0.000 ± 0.000	0.000 ± 0.000	2.97 ± 2.10	2.99 ± 2.08	11.2 ± 7.3	66.0 ± 41.2

*Dissolved nitrous oxide concentrations not included.

NA = not analyzed

Table 4. Gross Data for the Long-Term Inundated Experiment: Total Volume of Gas Produced in Aerobic Samples

Treatments [Brine/Bentonite]	Sample Designation	Volume of Gas Produced (ml) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (4)								
Formalin treated, w/o cellulose	4(NC)-f	5.93 ± 0.04	1.24 ± 2.02	1.43 ± 1.74	1.09 ± 1.05	-0.31 ± 0.00	NA	-2.17 ± 0.00
Formalin treated, with cellulose	4(C)-f	6.59 ± 0.16	4.38 ± 0.13	3.77 ± 0.13	1.82 ± 0.13	0.10 ± 0.03	NA	-1.98 ± 0.03
As is, w/o cellulose	4(NC)-a	5.43 ± 0.04	3.63 ± 0.53	4.78 ± 0.78	2.65 ± 0.45	1.92 ± 0.20	NA	-0.82 ± 0.20
As is, with cellulose	4(C)-a	5.17 ± 0.10	3.74 ± 0.10	2.99 ± 0.20	1.67 ± 0.24	1.26 ± 0.20	NA	-0.82 ± 0.27
Unamended/Inoculated (5)								
Formalin treated, w/o cellulose	5(NC)-f	5.08 ± 0.36	2.56 ± 0.83	1.19 ± 1.73	0.76 ± 0.94	0.54 ± 0.65	NA	-2.02 ± 0.36
Formalin treated, with cellulose	5(C)-f	5.65 ± 0.12	3.63 ± 0.03	2.72 ± 0.00	2.22 ± 0.00	0.88 ± 0.15	NA	-1.23 ± 0.12
As is, w/o cellulose	5(NC)-a	3.66 ± 0.30	3.62 ± 0.04	2.78 ± 0.08	1.64 ± 0.15	1.14 ± 0.11	NA	-2.06 ± 0.34
As is, with cellulose	5(C)-a	3.79 ± 0.06	3.13 ± 0.09	1.88 ± 0.19	0.16 ± 0.66	-1.94 ± 0.47	NA	-2.47 ± 0.78
Amended/Inoculated (6)								
As is, w/o cellulose	6(NC)-a	3.85 ± 0.04	0.84 ± 0.84	1.52 ± 0.84	1.56 ± 0.80	0.11 ± 0.53	-1.49 ± 0.34	-3.47 ± 0.15
As is, with cellulose	6(C)-a	2.60 ± 0.16	3.00 ± 0.06	5.07 ± 0.75	10.7 ± 1.8	14.9 ± 1.5	18.9 ± 1.1	18.4 ± 1.0
As is, with glucose	6(G)-a	1.60 ± 0.04	1.10 ± 0.69	0.69 ± 0.50	-0.34 ± 0.19	-1.07 ± 0.27	-1.62 ± 0.19	-1.52 ± 0.61
Excess nitrate, w/o cellulose	6(NC)-x	1.41 ± 0.08	-1.45 ± 0.04	-0.99 ± 0.08	0.00 ± 0.00	-0.27 ± 0.08	-1.30 ± 0.04	-3.05 ± 0.08
Excess nitrate, with cellulose	6(C)-x	2.91 ± 0.06	2.82 ± 0.09	6.79 ± 0.09	11.2 ± 1.2	18.7 ± 1.5	24.4 ± 0.9	27.3 ± 0.2
Excess nitrate, with glucose	6(G)-x	2.29 ± 0.23	2.51 ± 1.71	0.69 ± 0.57	-0.38 ± 0.5	-0.53 ± 0.11	-1.49 ± 0.15	-1.94 ± 0.69

NA = not analyzed

Table 5. Gross Data for the Long-Term Inundated Experiment: Production of Carbon Dioxide* in Aerobic Samples

Treatments [Brine/Bentonite]	Sample Designation	Carbon Dioxide (μmoles/sample) Incubation Time (Days)						
		0	45	69	104	132	164	200
Unamended/Uninoculated (4)								
Formalin treated, w/o cellulose	4(NC)-f	50.3 ± 0.4	88.8 ± 1.0	98.8 ± 0.4	101 ± 1	115 ± 2	NA	121 ± 4
Formalin treated, with cellulose	4(C)-f	71.1 ± 1.2	95.3 ± 0.2	110 ± 0	114 ± 0	127 ± 1	NA	137 ± 1
As is, w/o cellulose	4(NC)-a	17.7 ± 0.3	38.8 ± 0.4	44.5 ± 0.4	40.2 ± 0.3	42.7 ± 0.2	NA	40.0 ± 0.1
As is, with cellulose	4(C)-a	25.3 ± 1.5	47.6 ± 0.3	66.9 ± 11.8	49 ± 0.7	51.8 ± 0.5	NA	51.6 ± 0.1
Unamended/Inoculated (5)								
Formalin treated, w/o cellulose	5(NC)-f	37.3 ± 10.1	59.6 ± 15.7	66.1 ± 18.4	77.4 ± 18.1	76.0 ± 26.5	NA	79.0 ± 33.0
Formalin treated, with cellulose	5(C)-f	61.9 ± 0.9	83.5 ± 0.0	94.0 ± 0.2	93.2 ± 0.6	100 ± 0	NA	102 ± 0
As is, w/o cellulose	5(NC)-a	13.5 ± 2.9	36.7 ± 0.7	41.3 ± 0.2	38.7 ± 0.7	41.4 ± 0.7	NA	37.6 ± 0.7
As is, with cellulose	5(C)-a	23.7 ± 0.2	43.6 ± 0.4	61.3 ± 4.0	80.3 ± 2.1	101 ± 3	NA	145 ± 6
Amended/Inoculated (6)								
As is, w/o cellulose	6(NC)-a	11.3 ± 0.1	103 ± 3	169 ± 3	185 ± 2	201 ± 2	203 ± 0	181 ± 1
As is, with cellulose	6(C)-a	8.60 ± 0.00	72.4 ± 1.0	156 ± 5	247 ± 11	358 ± 19	492 ± 6	530 ± 6
As is, with glucose	6(G)-a	8.50 ± 2.80	67.1 ± 0.2	73.1 ± 0.4	72.9 ± 0.2	75.8 ± 0.4	79.5 ± 1.0	75.7 ± 1.7
Excess nitrate, w/o cellulose	6(NC)-x	10.5 ± 0.4	84.6 ± 0.0	153 ± 1	171 ± 3	139 ± 37	184 ± 4	159 ± 4
Excess nitrate, with cellulose	6(C)-x	8.9 ± 0.1	68.0 ± 1.2	164 ± 3	307 ± 23	499 ± 75	711 ± 46	741 ± 30
Excess nitrate, with glucose	6(G)-x	9.40 ± 0.90	65.0 ± 2.0	71.4 ± 1.2	71.2 ± 0.6	76.5 ± 0.2	80.1 ± 1.5	81.1 ± 5.0

*Dissolved carbon dioxide concentrations not included.

NA = not analyzed

Table 6. Gross Data for the Long-Term Inundated Experiment: Production of Nitrous Oxide* in Aerobic Samples

Treatments [Brine/Bentonite]	Sample Designation	Nitrous Oxide (µmoles/sample) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (4)							
Formalin treated, w/o cellulose	4(NC)-f	0.000 ± 0.000	0.002 ± 0.002	0.213 ± 0.146	0.010 ± 0.007	NA	0.000 ± 0.000
Formalin treated, with cellulose	4(C)-f	0.000 ± 0.000	0.008 ± 0.002	0.001 ± 0.001	0.019 ± 0.000	NA	0.006 ± 0.001
As is, w/o cellulose	4(NC)-a	0.000 ± 0.000	0.006 ± 0.001	0.003 ± 0.002	0.017 ± 0.000	NA	0.005 ± 0.003
As is, with cellulose	4(C)-a	0.000 ± 0.000	0.731 ± 0.489	0.188 ± 0.054	0.222 ± 0.070	NA	0.245 ± 0.101
Unamended/Inoculated (5)							
Formalin treated, w/o cellulose	5(NC)-f	0.000 ± 0.000	0.070 ± 0.049	0.009 ± 0.002	0.166 ± 0.106	NA	0.076 ± 0.049
Formalin treated, with cellulose	5(C)-f	0.000 ± 0.000	0.012 ± 0.001	0.011 ± 0.002	0.025 ± 0.001	NA	0.009 ± 0.002
As is, w/o cellulose	5(NC)-a	0.000 ± 0.000	0.038 ± 0.024	0.195 ± 0.065	0.210 ± 0.066	NA	0.161 ± 0.050
As is, with cellulose	5(C)-a	0.000 ± 0.000	0.010 ± 0.006	0.000 ± 0.000	0.000 ± 0.000	NA	0.064 ± 0.027
Amended/Inoculated (6)							
As is, w/o cellulose	6(NC)-a	0.000 ± 0.000	0.002 ± 0.001	1.21 ± 0.36	6.06 ± 4.21	4.96 ± 3.51	4.27 ± 2.55
As is, with cellulose	6(C)-a	0.000 ± 0.000	31.6 ± 13.2	160 ± 53	132 ± 8	0.528 ± 0.459	0.000 ± 0.000
As is, with glucose	6(G)-a	0.000 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.028 ± 0.001	0.007 ± 0.001	0.015 ± 0.004
Excess nitrate, w/o cellulose	6(NC)-x	0.000 ± 0.000	0.000 ± 0.000	14.6 ± 2.7	13.9 ± 5.2	13.8 ± 5.4	11.6 ± 4.9
Excess nitrate, with cellulose	6(C)-x	0.000 ± 0.000	27.1 ± 6.5	142 ± 22	217 ± 41	354 ± 41	425 ± 35
Excess nitrate, with glucose	6(G)-x	0.000 ± 0.000	0.005 ± 0.001	0.010 ± 0.001	0.022 ± 0.003	0.092 ± 0.063	0.011 ± 0.001

*Dissolved nitrous oxide concentrations not included.

NA = not analyzed

Table 7. Gross Data for the Long-Term Inundated Experiment: Total Volume of Gas Produced in Anaerobic Samples

Treatments [Brine]	Sample Designation	Volume of Gas Produced (ml) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (7)								
Formalin treated, w/o cellulose	7(NC)-f	2.99 ± 0.10	2.65 ± 0.10	2.01 ± 0.03	1.09 ± 0.06	1.66 ± 0.00	NA	0.06 ± 0.03
Formalin treated, with cellulose	7(C)-f	4.28 ± 0.06	3.49 ± 0.06	3.10 ± 0.03	1.95 ± 0.03	2.37 ± 0.03	NA	1.37 ± 0.00
As is, w/o cellulose	7(NC)-a	3.20 ± 0.34	2.96 ± 0.07	0.99 ± 0.14	2.24 ± 0.00	2.07 ± 0.10	NA	0.65 ± 0.31
As is, with cellulose	7(C)-a	3.37 ± 0.27	3.47 ± 0.10	2.99 ± 0.07	1.33 ± 0.34	2.28 ± 1.17	NA	0.20 ± 0.17
Unamended/Inoculated (8)								
Formalin treated, w/o cellulose	8(NC)-f	3.79 ± 0.04	2.31 ± 0.07	1.73 ± 0.18	0.76 ± 0.11	1.55 ± 0.11	NA	-0.07 ± 0.11
Formalin treated, with cellulose	8(C)-f	3.89 ± 0.03	3.39 ± 0.09	2.54 ± 0.15	1.58 ± 0.00	2.14 ± 0.06	NA	1.02 ± 0.03
As is, w/o cellulose	8(NC)-a	3.66 ± 0.08	-1.37 ± 0.99	1.41 ± 0.23	1.87 ± 0.08	2.10 ± 0.08	NA	-0.50 ± 0.00
As is, with cellulose	8(C)-a	3.47 ± 0.06	3.63 ± 0.13	2.53 ± 0.16	1.78 ± 0.13	2.22 ± 0.06	NA	2.44 ± 0.63
Amended/Inoculated (9)								
As is, w/o cellulose	9(NC)-a	3.77 ± 0.04	3.35 ± 0.08	6.63 ± 0.30	7.96 ± 1.33	9.07 ± 0.88	8.41 ± 0.48	8.04 ± 0.46
As is, with cellulose	9(C)-a	3.35 ± 0.09	3.44 ± 0.16	4.04 ± 0.03	11.3 ± 0.5	16.7 ± 1.3	19.2 ± 1.2	19.4 ± 0.6
As is, with glucose	9(G)-a	2.97 ± 0.08	2.59 ± 0.15	2.36 ± 0.15	1.64 ± 0.15	1.52 ± 0.27	0.18 ± 0.16	-1.26 ± 0.30
Excess nitrate, w/o cellulose	9(NC)-x	3.24 ± 0.04	2.86 ± 0.08	4.30 ± 0.23	6.59 ± 0.76	9.18 ± 0.23	8.65 ± 0.67	6.93 ± 0.27
Excess nitrate, with cellulose	9(C)-x	3.29 ± 0.13	2.28 ± 0.38	5.76 ± 1.47	16.6 ± 2.9	14.2 ± 3.9	28.9 ± 6.2	34.1 ± 7.1
Excess nitrate, with glucose	9(G)-x	3.01 ± 0.11	0.61 ± 0.15	0.19 ± 0.11	0.46 ± 0.04	0.46 ± 0.61	1.33 ± 2.08	1.14 ± 2.90

NA = not analyzed

Table 8. Gross Data for the Long-Term Inundated Experiment: Production of Carbon Dioxide* in Anaerobic Samples

Treatments [Brine]	Sample Designation	Carbon Dioxide (µmoles/sample) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (7)								
Formalin treated, w/o cellulose	7(NC)-f	2.26 ± 0.01	1.92 ± 0.03	2.20 ± 0.20	2.24 ± 0.04	2.33 ± 0.01	NA	2.39 ± 0.01
Formalin treated, with cellulose	7(C)-f	31.7 ± 0.5	38.0 ± 0.1	38.0 ± 0.1	35.0 ± 0.2	36.4 ± 0.1	NA	36.3 ± 0.1
As is, w/o cellulose	7(NC)-a	1.12 ± 0.01	0.58 ± 0.13	0.90 ± 0.00	1.37 ± 0.01	1.35 ± 0.01	NA	1.46 ± 0.03
As is, with cellulose	7(C)-a	13.0 ± 0.4	19.3 ± 0.1	20.5 ± 0.1	19.5 ± 0.1	20.5 ± 0.2	NA	19.4 ± 0.2
Unamended/Inoculated (8)								
Formalin treated, w/o cellulose	8(NC)-f	4.42 ± 0.01	3.88 ± 0.08	3.89 ± 0.13	3.88 ± 0.04	3.99 ± 0.03	NA	3.97 ± 0.03
Formalin treated, with cellulose	8(C)-f	32.1 ± 0.7	34.3 ± 0.4	33.5 ± 0.2	31.2 ± 0.0	32.5 ± 0.5	NA	32.8 ± 0.4
As is, w/o cellulose	8(NC)-a	1.96 ± 0.02	1.37 ± 0.01	2.29 ± 0.02	2.75 ± 0.01	2.86 ± 0.01	NA	2.74 ± 0.01
As is, with cellulose	8(C)-a	12.5 ± 0.2	18.4 ± 0.2	19.0 ± 0.1	17.8 ± 0.7	22.7 ± 0.5	NA	30.1 ± 1.7
Amended/Inoculated (9)								
As is, w/o cellulose	9(NC)-a	3.02 ± 0.00	6.86 ± 0.02	58.9 ± 3.1	95.9 ± 9.5	109 ± 4	119 ± 3	120 ± 1
As is, with cellulose	9(C)-a	2.70 ± 0.00	25.8 ± 0.2	42.5 ± 1.7	132 ± 3	200 ± 7	240 ± 3	250 ± 4
As is, with glucose	9(G)-a	2.25 ± 0.01	3.36 ± 0.04	2.96 ± 0.04	3.50 ± 0.05	3.88 ± 0.06	4.26 ± 0.09	4.31 ± 0.07
Excess nitrate, w/o cellulose	9(NC)-x	3.07 ± 0.03	5.17 ± 0.28	35.3 ± 5.3	94.5 ± 4.4	120 ± 1	125 ± 1	123 ± 1
Excess nitrate, with cellulose	9(C)-x	5.40 ± 0.00	26.6 ± 0.2	65.8 ± 17.1	193 ± 33	249 ± 32	352 ± 40	430 ± 41
Excess nitrate, with glucose	9(G)-x	1.81 ± 0.27	2.88 ± 0.19	2.65 ± 0.25	8.64 ± 3.62	23.6 ± 13.3	53.6 ± 31.4	74.9 ± 40.9

*Dissolved carbon dioxide concentrations not included.
NA = not analyzed

Table 9. Gross Data for the Long-Term Inundated Experiment: Production of Nitrous Oxide* in Anaerobic Samples

Treatments [Brine]	Sample Designation	Nitrous Oxide (μmoles/sample) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (7)							
Formalin treated, w/o cellulose	7(NC)-f	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
Formalin treated, with cellulose	7(C)-f	0.000 ± 0.000	0.009 ± 0.007	0.004 ± 0.001	0.007 ± 0.005	NA	0.002 ± 0.002
As is, w/o cellulose	7(NC)-a	0.000 ± 0.000	0.003 ± 0.002	0.001 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
As is, with cellulose	7(C)-a	0.000 ± 0.000	0.002 ± 0.002	0.003 ± 0.002	0.000 ± 0.000	NA	0.002 ± 0.002
Unamended/Inoculated (8)							
Formalin treated, w/o cellulose	8(NC)-f	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
Formalin treated, with cellulose	8(C)-f	0.000 ± 0.000	0.002 ± 0.001	0.002 ± 0.000	0.014 ± 0.001	NA	0.008 ± 0.000
As is, w/o cellulose	8(NC)-a	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
As is, with cellulose	8(C)-a	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
Amended/Inoculated (9)							
As is, w/o cellulose	9(NC)-a	0.000 ± 0.000	0.376 ± 0.262	35.5 ± 25.1	7.55 ± 5.34	0.000 ± 0.000	0.359 ± 0.235
As is, with cellulose	9(C)-a	0.000 ± 0.000	0.359 ± 0.145	13.2 ± 10.1	86.5 ± 41.3	89.3 ± 43.0	77.8 ± 42.2
As is, with glucose	9(G)-a	0.000 ± 0.000	0.002 ± 0.001	0.000 ± 0.000	0.005 ± 0.003	0.005 ± 0.000	0.015 ± 0.010
Excess nitrate, w/o cellulose	9(NC)-x	0.000 ± 0.000	0.081 ± 0.021	5.13 ± 2.48	0.014 ± 0.002	0.000 ± 0.000	0.000 ± 0.000
Excess nitrate, with cellulose	9(C)-x	0.000 ± 0.000	0.870 ± 0.422	85.7 ± 50.2	196 ± 75	280 ± 160	395 ± 228
Excess nitrate, with glucose	9(G)-x	0.000 ± 0.000	0.028 ± 0.020	3.84 ± 2.72	0.523 ± 0.006	0.539 ± 0.381	13.8 ± 0.5

*Dissolved nitrous oxide concentrations not included.

NA = not analyzed

Table 10. Gross Data for the Long-Term Inundated Experiment: Total Volume of Gas Produced in Anaerobic Samples.

Treatments* [Brine/Bentonite]	Sample Designation	Volume of Gas Produced (ml) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (10)								
Formalin treated, w/o cellulose	10(NC)-f	4.65 ± 0.23	4.61 ± 0.47	2.37 ± 1.47	3.41 ± 0.19	4.07 ± 0.04	NA	2.33 ± 0.00
Formalin treated, with cellulose	10(C)-f	4.96 ± 0.06	4.64 ± 0.03	4.22 ± 0.00	2.94 ± 0.16	3.39 ± 0.16	NA	2.05 ± 0.16
As is, w/o cellulose	10(NC)-a	4.24 ± 0.33	4.04 ± 0.04	3.51 ± 0.69	2.86 ± 0.08	3.27 ± 0.20	NA	1.55 ± 0.08
As is, with cellulose	10(C)-a	3.84 ± 0.48	3.84 ± 0.14	3.33 ± 0.27	2.01 ± 0.41	2.18 ± 0.48	NA	0.14 ± 0.44
Unamended/Inoculated (11)								
Formalin treated, w/o cellulose	11(NC)-f	5.16 ± 0.07	5.01 ± 0.14	3.89 ± 0.22	2.85 ± 0.25	3.57 ± 0.25	NA	1.23 ± 0.22
Formalin treated, with cellulose	11(C)-f	4.65 ± 0.03	4.53 ± 0.00	3.66 ± 0.03	1.73 ± 1.14	2.16 ± 1.08	NA	0.94 ± 1.20
As is, w/o cellulose	11(NC)-a	4.23 ± 0.11	3.62 ± 0.08	3.47 ± 0.19	2.06 ± 0.19	2.44 ± 0.30	NA	0.34 ± 0.34
As is, with cellulose	11(C)-a	4.38 ± 0.03	4.19 ± 0.06	3.19 ± 0.16	2.88 ± 0.22	3.88 ± 0.19	NA	4.38 ± 0.38
Amended/Inoculated (12)								
As is, w/o cellulose	12(NC)-a	3.85 ± 0.08	4.53 ± 0.15	6.97 ± 0.15	9.90 ± 0.08	10.9 ± 0.1	11.1 ± 0.3	9.87 ± 0.80
As is, with cellulose	12(C)-a	3.32 ± 0.25	4.29 ± 0.09	7.92 ± 0.44	16.8 ± 0.4	19.8 ± 0.4	18.3 ± 0.3	19.5 ± 0.4
As is, with glucose	12(G)-a	3.50 ± 0.04	5.52 ± 0.34	9.49 ± 2.40	24.7 ± 3.3	33.9 ± 0.6	38.8 ± 1.8	37.8 ± 2.3
Excess nitrate, w/o cellulose	12(NC)-x	3.66 ± 0.00	4.19 ± 0.15	8.19 ± 0.69	11.8 ± 0.3	12.4 ± 0.0	12.8 ± 0.1	11.2 ± 0.1
Excess nitrate, with cellulose	12(C)-x	3.38 ± 0.09	3.76 ± 0.16	9.36 ± 0.31	15.7 ± 0.4	20.8 ± 0.5	23.8 ± 0.7	28.9 ± 1.4
Excess nitrate, with glucose	12(G)-x	3.09 ± 0.08	2.29 ± 0.19	2.21 ± 0.46	23.4 ± 0.6	52.3 ± 6.7	77.4 ± 20.2	80.7 ± 21.1

NA = not analyzed

Table 11. Gross Data for the Long-Term Inundated Experiment: Production of Carbon Dioxide* in Anaerobic Samples

Treatments [Brine/Bentonite]	Sample Designation	Carbon Dioxide (µmoles/sample)						
		0	45	69		132	164	200
Unamended/Uninoculated (10)								
Formalin treated, w/o cellulose	10(NC)-f	55.8 ± 0.6	82.9 ± 1.1	85.5 ± 0.8	86.4 ± 1.7	92.8 ± 2.0	NA	94.9 ± 1.1
Formalin treated, with cellulose	10(C)-f	65.5 ± 1.0	85.5 ± 0.2	88.6 ± 0.4	81.9 ± 0.3	88.7 ± 0.9	NA	90.2 ± 0.3
As is, w/o cellulose	10(NC)-a	16.4 ± 0.4	37.7 ± 0.0	38.4 ± 0.1	38.1 ± 0.3	39.8 ± 0.3	NA	40.0 ± 0.4
As is, with cellulose	10(C)-a	26.6 ± 0.5	42.6 ± 0.2	43.0 ± 0.4	41.3 ± 0.2	43.1 ± 0.5	NA	41.1 ± 0.2
Unamended/Inoculated (11)								
Formalin treated, w/o cellulose	11(NC)-f	53.0 ± 0.2	76.9 ± 0.6	80.2 ± 0.4	76.5 ± 0.0	83.7 ± 0.6	NA	83.5 ± 0.2
Formalin treated, with cellulose	11(C)-f	64.4 ± 1.3	76.3 ± 0.5	78.6 ± 0.2	75.1 ± 1.9	78.6 ± 1.6	NA	80.4 ± 0.8
As is, w/o cellulose	11(NC)-a	20.8 ± 0.0	35.0 ± 0.1	36.3 ± 0.1	35.2 ± 0.2	19.6 ± 11.9	NA	36.2 ± 0.2
As is, with cellulose	11(C)-a	30.1 ± 0.6	38.1 ± 0.2	40.5 ± 0.1	48 ± 2.5	59.9 ± 0.7	NA	77.6 ± 1.0
Amended/Inoculated (12)								
As is, w/o cellulose	12(NC)-a	14.0 ± 0.0	60.2 ± 0.2	99.8 ± 4.7	150 ± 4	16.6 ± 0.4	177 ± 1	179 ± 0
As is, with cellulose	12(C)-a	12.0 ± 0.8	55.0 ± 0.3	104 ± 5	209 ± 2	260 ± 8	295 ± 10	338 ± 10
As is, with glucose	12(G)-a	15.2 ± 0.6	76.0 ± 2.4	144 ± 28	375 ± 36	590 ± 15	691 ± 20	786 ± 25
Excess nitrate, w/o cellulose	12(NC)-x	13.9 ± 0.3	69.2 ± 0.0	121 ± 3	166 ± 1	186 ± 2	196 ± 2	196 ± 2
Excess nitrate, with cellulose	12(C)-x	10.3 ± 0.6	57.4 ± 1.7	122 ± 3	195 ± 5	264 ± 6	309 ± 7	371 ± 14
Excess nitrate, with glucose	12(G)-x	15.1 ± 0.0	69.4 ± 1.2	111 ± 4	422 ± 12	916 ± 35	1370 ± 19	1610 ± 43

*Dissolved carbon dioxide concentrations not included.

NA = not analyzed

Table 12. Gross Data for the Long-Term Inundated Experiment: Production of Nitrous Oxide* in Anaerobic Samples

Treatments [Brine/Bentonite]	Sample Designation	Nitrous Oxide (μmoles/sample) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (10)							
Formalin treated, w/o cellulose	10(NC)-f	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
Formalin treated, with cellulose	10(C)-f	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
As is, w/o cellulose	10(NC)-a	0.000 ± 0.000	0.004 ± 0.003	0.102 ± 0.065	1.040 ± 0.490	NA	1.73 ± 0.10
As is, with cellulose	10(C)-a	0.000 ± 0.000	0.005 ± 0.002	0.008 ± 0.004	0.021 ± 0.009	NA	0.031 ± 0.011
Unamended/Inoculated (11)							
Formalin treated, w/o cellulose	11(NC)-f	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA	0.002 ± 0.001
Formalin treated, with cellulose	11(C)-f	0.000 ± 0.000	0.000 ± 0.000	0.006 ± 0.002	0.024 ± 0.003	NA	0.000 ± 0.000
As is, w/o cellulose	11(NC)-a	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.037 ± 0.026	NA	0.007 ± 0.005
As is, with cellulose	11(C)-a	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	NA	0.000 ± 0.000
Amended/Inoculated (12)							
As is, w/o cellulose	12(NC)-a	0.000 ± 0.000	0.016 ± 0.006	0.534 ± 0.378	0.000 ± 0.000	0.000 ± 0.000	3.35 ± 2.19
As is, with cellulose	12(C)-a	0.000 ± 0.000	0.111 ± 0.056	2.34 ± 1.92	0.000 ± 0.000	0.301 ± 0.246	0.000 ± 0.000
As is, with glucose	12(G)-a	0.000 ± 0.000	1.56 ± 1.10	40.1 ± 28.3	0.782 ± 0.557	0.016 ± 0.011	0.000 ± 0.000
Excess nitrate, w/o cellulose	12(NC)-x	0.000 ± 0.000	0.007 ± 0.001	0.081 ± 0.057	0.000 ± 0.000	0.003 ± 0.002	0.481 ± 0.340
Excess nitrate, with cellulose	12(C)-x	0.004 ± 0.000	3.80 ± 2.39	0.022 ± 0.018	47.1 ± 17.8	154 ± 20	311 ± 23
Excess nitrate, with glucose	12(G)-x	0.005 ± 0.000	0.077 ± 0.049	2.31 ± 1.59	261 ± 184	616 ± 428	585 ± 406

*Dissolved nitrous oxide concentrations not included.

NA = not analyzed

Table 13. Total Volume of Gas Produced in Aerobic Samples in the Presence of Cellulose*

Treatments [Brine]	Sample Desig.	Milliliters of Gas Produced/Gram Cellulose Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (1)								
Formalin	1(C)-f	0.24 ± 0.06	0.89 ± 0.03	0.85 ± 0.02	0.42 ± 0.21	0.27 ± 0.19	NA	0.42 ± 0.18
As is	1(C)-a	0.05 ± 0.02	-0.29 ± 0.19	-0.36 ± 0.18	-0.31 ± 0.14	-0.20 ± 0.12	NA	-0.18 ± 0.10
Unamended/Inoculated (2)								
Formalin	2(C)-f	-0.01 ± 0.01	-0.03 ± 0.08	-0.35 ± 0.11	-0.32 ± 0.08	-0.41 ± 0.11	NA	-0.11 ± 0.09
As is	2(C)-a	-0.08 ± 0.01	0.01 ± 0.04	-0.15 ± 0.03	-0.11 ± 0.04	0.00 ± 0.05	NA	-0.34 ± 0.07
Amended/Inoculated (3)								
As is	3(C)-a	-0.12 ± 0.03	-0.27 ± 0.14	-0.25 ± 0.06	0.36 ± 0.31	0.29 ± 0.24	0.07 ± 0.13	0.86 ± 0.08
Exc. nitrate	3(C)-x	-0.02 ± 0.01	0.02 ± 0.14	1.39 ± 0.61	2.38 ± 0.87	3.10 ± 1.06	4.02 ± 1.21	4.42 ± 0.80

* All values have been corrected with specific controls for gas production in the absence of cellulose
NA = not analyzed

Table 14. Total Volume of Gas Produced in Aerobic Samples in the Presence of Cellulose and Bentonite*

Treatments [Brine/Bent.]	Sample Designation	Milliliters of Gas Produced/Gram Cellulose Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (4)								
Formalin	4(C)-f	0.13 ± 0.03	0.63 ± 0.03	0.47 ± 0.03	0.15 ± 0.03	0.02 ± 0.01	NA	0.04 ± 0.01
As is	4(C)-a	-0.05 ± 0.02	0.02 ± 0.02	-0.36 ± 0.04	-0.20 ± 0.05	-0.13 ± 0.04	NA	0.00 ± 0.05
Unamended/Inoculated (5)								
Formalin	5(C)-f	0.11 ± 0.02	0.21 ± 0.01	0.31 ± 0.00	0.29 ± 0.00	0.07 ± 0.03	NA	0.16 ± 0.02
As is	5(C)-a	0.03 ± 0.01	-0.10 ± 0.02	-0.18 ± 0.04	-0.30 ± 0.13	-0.23 ± 0.09	NA	-0.08 ± 0.16
Amended/Inoculated (6)								
As is	6(C)-a	-0.25 ± 0.03	0.43 ± 0.01	0.71 ± 0.15	1.82 ± 0.35	2.96 ± 0.30	4.07 ± 0.22	4.38 ± 0.20
Exc. nitrate	6(C)-x	0.30 ± 0.01	0.85 ± 0.02	1.56 ± 0.02	2.23 ± 0.24	3.74 ± 0.29	5.15 ± 0.18	6.07 ± 0.04

* All values have been corrected with specific controls for gas production in the absence of cellulose
NA = not analyzed

Table 15. Production of Carbon Dioxide in Aerobic Samples in the Presence of Cellulose*

Treatments [Brine]	Sample Desig.	Carbon Dioxide (µmoles/gram cellulose) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (1)								
Formalin	1(C)-f	5.58 ± 0.54	7.66 ± 0.02	8.20 ± 0.04	7.46 ± 0.16	7.64 ± 0.06	NA	7.62 ± 0.10
As is	1(C)-a	2.48 ± 0.10	3.92 ± 0.06	4.21 ± 0.02	3.93 ± 0.02	4.25 ± 0.02	NA	4.00 ± 0.02
Unamended/Inoculated (2)								
Formalin	2(C)-f	5.91 ± 0.16	6.24 ± 0.00	6.80 ± 0.14	5.88 ± 0.12	6.04 ± 0.02	NA	5.65 ± 0.22
As is	2(C)-a	1.96 ± 0.06	3.35 ± 0.16	3.94 ± 0.18	4.98 ± 0.18	6.87 ± 0.20	NA	8.30 ± 0.28
Amended/Inoculated (3)								
As is	3(C)-a	ND	4.62 ± 0.28	3.78 ± 1.18	20.4 ± 7.4	29.6 ± 5	36.6 ± 4.2	40.8 ± 5.4
Exc. nitrate	3(C)-x	ND	6.88 ± 0.00	32.2 ± 4.2	61.4 ± 3.6	83.6 ± 2.6	96.2 ± 4.0	95.6 ± 6.0

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected

Table 16. Production of Carbon Dioxide in Aerobic Samples in the Presence of Cellulose and Bentonite*

Treatments [Brine/Bent.]	Sample Desig.	Carbon Dioxide (μmoles/gram cellulose) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (4)								
Formalin	4(C)-f	4.16 ± 0.24	1.30 ± 0.04	2.24 ± 0.00	2.60 ± 0.00	2.40 ± 0.20	NA	3.20 ± 0.20
As is	4(C)-a	1.52 ± 0.30	1.76 ± 0.06	4.48 ± 2.36	1.76 ± 0.14	1.82 ± 0.10	NA	2.32 ± 0.02
Unamended/Inoculated (5)								
Formalin	5(C)-f	4.92 ± 0.18	4.78 ± 0.00	5.58 ± 0.04	3.16 ± 0.12	4.80 ± 0.00	NA	4.60 ± 0.00
As is	5(C)-a	2.04 ± 0.04	1.38 ± 0.08	4.00 ± 0.80	8.32 ± 0.42	11.9 ± 0.6	NA	21.5 ± 1.2
Amended/Inoculated (6)								
As is	6(C)-a	ND	ND	ND	12.4 ± 2.2	31.4 ± 3.8	57.8 ± 1.2	69.8 ± 1.2
Exc. nitrate	6(C)-x	ND	ND	2.20 ± 0.60	27.2 ± 4.6	72.0 ± 15.0	105 ± 9	116 ± 6

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected

Table 17. Production of Nitrous Oxide in Aerobic Samples in the Presence of Cellulose*

Treatments [Brine]	Sample Desig.	Nitrous Oxide (μmoles/gram cellulose) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (1)							
Formalin	1(C)-f	ND	ND	0.001 ± 0.000	0.003 ± 0.000	NA	0.001 ± 0.000
As is	1(C)-a	ND	ND	0.001 ± 0.000	0.003 ± 0.000	NA	ND
Unamended/Inoculated (2)							
Formalin	2(C)-f	ND	ND	ND	0.003 ± 0.000	NA	0.001 ± 0.000
As is	2(C)-a	0.002 ± 0.000	ND	ND	ND	NA	ND
Amended/Inoculated (3)							
As is	3(C)-a	0.001 ± 0.000	0.024 ± 0.029	23.6 ± 9.6	24.4 ± 7.8	1.76 ± 1.20	5.83 ± 5.04
Exc. nitrate	3(C)-x	ND	5.64 ± 1.77	49.0 ± 2.2	65.2 ± 3.6	96.8 ± 13.4	115 ± 26

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected
NA - not analyzed

Table 18. Production of Nitrous Oxide in Aerobic Samples in the Presence of Cellulose and Bentonite*

Treatments [Brine/Bent.]	Sample Desig.	Nitrous Oxide (μmoles/gram cellulose) Incubation Time (Days)					
		0	69			164	200
Unamended/Uninoculated (4)							
Formalin	4(C)-f	ND	0.001 ± 0.000	ND	0.002 ± 0.000	NA	0.001 ± 0.000
As is	4(C)-a	ND	0.145 ± 0.098	0.037 ± 0.011	0.041 ± 0.014	NA	0.048 ± 0.020
Unamended/Inoculated (5)							
Formalin	5(C)-f	ND	ND	ND	ND	NA	ND
As is	5(C)-a	ND	0.000 ± 0.001	ND	ND	NA	ND
Amended/Inoculated (6)							
As is	6(C)-a	ND	6.32 ± 2.64	31.8 ± 10.6	25.2 ± 1.6	ND	ND
Exc. nitrate	6(C)-x	ND	5.42 ± 1.30	25.5 ± 4.4	40.6 ± 8.2	68.0 ± 8.2	82.7 ± 7.0

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected
NA - not analyzed

Table 19. Total Volume of Gas Produced in Anaerobic Samples in the Presence of Cellulose*

Treatments [Brine]	Sample Designation	Milliliters of Gas Produced/Gram Cellulose Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (7)								
Formalin	7(C)-f	0.26 ± 0.01	0.17 ± 0.01	0.22 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	NA	0.26 ± 0.00
As is	7(C)-a	0.03 ± 0.05	0.10 ± 0.02	0.40 ± 0.01	-0.18 ± 0.07	0.04 ± 0.23	NA	-0.09 ± 0.03
Unamended/Inoculated (8)								
Formalin	8(C)-f	0.02 ± 0.01	0.22 ± 0.02	0.16 ± 0.03	0.16 ± 0.00	0.12 ± 0.01	NA	0.22 ± 0.00
As is	8(C)-a	-0.04 ± 0.01	1.00 ± 0.03	0.22 ± 0.03	-0.02 ± 0.03	0.02 ± 0.01	NA	0.59 ± 0.13
Amended/Inoculated (9)								
As is	9(C)-a	-0.08 ± 0.02	0.02 ± 0.03	-0.52 ± 0.01	0.66 ± 0.09	1.52 ± 0.25	2.15 ± 0.24	2.27 ± 0.12
Exc. nitrate	9(C)-x	0.01 ± 0.03	-0.12 ± 0.08	0.29 ± 0.29	2.00 ± 0.58	2.24 ± 0.78	4.04 ± 1.24	5.44 ± 1.42

* All values have been corrected with specific controls for gas production in the absence of cellulose
NA = not analyzed

Table 20. Total Volume of Gas Produced in Anaerobic Samples in the Presence of Cellulose and Bentonite*

Treatments [Brine/Bent.]	Sample Designation	Milliliters of Gas Produced/Gram Cellulose						
		Incubation Time (Days)						
		0	45	69	104	132	164	200
Unamended/Uninoculated (10)								
Formalin	10(C)-f	0.06 ± 0.01	0.01 ± 0.01	0.37 ± 0.00	-0.09 ± 0.03	-0.14 ± 0.03	NA	-0.06 ± 0.03
As is	10(C)-a	-0.08 ± 0.10	-0.04 ± 0.03	-0.04 ± 0.05	-0.17 ± 0.08	-0.22 ± 0.10	NA	-0.28 ± 0.09
Unamended/Inoculated (11)								
Formalin	11(C)-f	-0.10 ± 0.01	-0.10 ± 0.00	-0.05 ± 0.01	-0.22 ± 0.23	-0.28 ± 0.22	NA	-0.06 ± 0.24
As is	11(C)-a	0.03 ± 0.01	0.11 ± 0.01	-0.06 ± 0.03	0.16 ± 0.04	0.29 ± 0.04	NA	0.81 ± 0.08
Amended/Inoculated (12)								
As is	12(C)-a	-0.11 ± 0.05	-0.05 ± 0.02	0.19 ± 0.09	1.39 ± 0.09	1.78 ± 0.08	1.44 ± 0.07	1.92 ± 0.08
Exc. nitrate	12(C)-x	-0.06 ± 0.02	-0.09 ± 0.03	0.23 ± 0.06	0.78 ± 0.09	1.68 ± 0.10	2.19 ± 0.14	3.52 ± 0.28

* All values have been corrected with specific controls for gas production in the absence of cellulose
NA = not analyzed

Table 21. Production of Carbon Dioxide in Anaerobic Samples in the Presence of Cellulose*

Treatments [Brine]	Sample Desig.	Carbon Dioxide (μmoles/gram cellulose) Incubation Time (Days) 0	45	69	104	132	164	200
Unamended/Uninoculated (7)								
Formalin	7(C)-f	5.89 ± 0.10	7.22 ± 0.02	7.16 ± 0.02	6.55 ± 0.04	6.81 ± 0.02	NA	6.78 ± 0.02
As is	7(C)-a	2.38 ± 0.08	3.74 ± 0.02	3.92 ± 0.02	3.63 ± 0.02	3.83 ± 0.04	NA	3.59 ± 0.04
Unamended/Inoculated (8)								
Formalin	8(C)-f	5.54 ± 0.14	6.08 ± 0.08	5.92 ± 0.04	5.46 ± 0.00	5.70 ± 0.10	NA	5.77 ± 0.08
As is	8(C)-a	2.11 ± 0.04	3.41 ± 0.04	3.34 ± 0.02	3.01 ± 0.14	3.97 ± 0.10	NA	5.47 ± 0.34
Amended/Inoculated (9)								
As is	9(C)-a	ND	3.79 ± 0.04	ND	7.22 ± 0.60	18.2 ± 1.4	24.2 ± 0.6	26.0 ± 0.8
Exc. nitrate	9(C)-x	0.47 ± 0.00	4.29 ± 0.04	6.10 ± 3.42	19.7 ± 6.6	25.8 ± 6.4	45.4 ± 8.0	61.4 ± 8.2

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected

Table 22. Production of Carbon Dioxide in Anaerobic Samples in the Presence of Cellulose and Bentonite*

Treatments [Brine/Bent.]	Sample Desig.	Carbon Dioxide (µmoles/gram cellulose) Incubation Time (Days)						
		0	45	69	104	132	164	200
Unamended/Uninoculated (10)								
Formalin	10(C)-f	1.94 ± 0.2	0.52 ± 0.04	0.62 ± 0.08	ND	ND	NA	ND
As is	10(C)-a	2.04 ± 0.1	0.98 ± 0.04	0.92 ± 0.08	0.64 ± 0.04	0.66 ± 0.10	NA	0.22 ± 0.04
Unamended/Inoculated (11)								
Formalin	11(C)-f	2.28 ± 0.26	ND	ND	ND	ND	NA	ND
As is	11(C)-a	1.86 ± 0.12	0.62 ± 0.04	0.84 ± 0.02	2.56 ± 0.50	8.06 ± 0.14	NA	8.28 ± 0.20
Amended/Inoculated (12)								
As is	12(C)-a	ND	ND	0.84 ± 1.00	11.8 ± 0.4	48.68 ± 1.6	23.6 ± 2.0	31.8 ± 2.0
Exc. nitrate	12(C)-x	ND	ND	0.20 ± 0.60	5.80 ± 1.00	15.6 ± 1.2	22.6 ± 1.4	35.0 ± 2.8

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected

Table 23. Production of Nitrous Oxide in Anaerobic Samples in the Presence of Cellulose*

Treatments [Brine]	Sample Desig.	Nitrous Oxide (μmoles/gram cellulose) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (7)							
Formalin	7(C)-f	ND	0.002 ± 0.001	0.001 ± 0.000	0.001 ± 0.001	NA	ND
As is	7(C)-a	ND	ND	ND	ND	NA	ND
Unamended/Inoculated (8)							
Formalin	8(C)-f	ND	ND	ND	0.003 ± 0.000	NA	0.002 ± 0.000
As is	8(C)-a	ND	ND	ND	ND	NA	ND
Amended/Inoculated (9)							
As is	9(C)-a	ND	ND	ND	15.8 ± 8.3	17.9 ± 8.6	15.5 ± 8.4
Exc. nitrate	9(C)-x	ND	0.158 ± 0.084	16.1 ± 10.0	39.2 ± 15.0	56.0 ± 32.0	79.0 ± 45.6

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected
NA - not analyzed

Table 24. Production of Nitrous Oxide* in Anaerobic Samples in the Presence of Cellulose and Bentonite

Treatments [Brine/Bent.]	Sample Desig.	Nitrous Oxide (μmoles/gram cellulose) Incubation Time (Days) 0	69	104	132	164	200
Unamended/Uninoculated (10)							
Formalin	10(C)-f	ND	ND	ND	ND	NA	ND
As is	10(C)-a	ND	ND	ND	ND	NA	ND
Unamended/Inoculated (11)							
Formalin	11(C)-f	ND	ND	0.001 ± 0.000	0.005 ± 0.001	NA	ND
As is	11(C)-a	ND	ND	ND	ND	NA	ND
Amended/Inoculated (12)							
As is	12(C)-a	ND	0.019 ± 0.011	0.361 ± 0.384	ND	0.060 ± 0.049	ND
Exc. nitrate	12(C)-x	0.001 ± 0.000	0.759 ± 0.478	0.000 ± 0.000	9.42 ± 3.56	30.8 ± 4.0	62.1 ± 4.6

* All values have been corrected with specific controls for gas production in the absence of cellulose; dissolved gas concentration not included
ND - not detected
NA - not analyzed

APPENDIX E: MEASUREMENT OF MIXED INOCULUM ACTIVITY

APPENDIX MEASUREMENTS OF MIXED INOCULUM ACTIVITY

To determine the activity of the mixed inoculum, brine samples were incubated ith glucose with an initial air and N. atmosphere. Total gas, CO_2 and N_2O were periodically monitored.

Aerobic Samples

otal Gas Production

Samples containing glucose, nutrients, excess nitrates with and without bentonite did not show an increase in total gas production (Figure 25 Tables 1( )-2(a) Appendix D

Carbon Dioxide Production

Production of carbon dioxide evident after 132 days in amended and in amended plus nitrate samples without bentonite. After 200 days, amended samples produced 323 μmol of CO_2, whereas amended plus excess nitrate samples produced 296 μmol CO_2. Samples with bentonite did produce significant amounts of CO_2 beyond the initial background level 70-80 μmol (Figure 26, Tables 1(b)-2(b) Appendix D

Nitrous Oxide Production

Nitrous oxide was detected at 132 days in aerobic samples without bentonite (Figure 27 Tables)-2(Appendix D Less N.O was detected in amended samples with nitrate, and in samples containing bentonite, N_2O not detected.

Anaerobic Samples

Total Gas Production

Amended and amended samples with excess nitrate showed no increase in total gas production. However, in the presence of bentonite, production in amended samples increased after 45 days of incubation and reached to 37.8 mL at 200 days (Figure 28, Tables 3(a)-4(a) Appendix D). Amended samples plus excess nitrate produced more gas (80.65 mL of gas at 200 days) than the other samples.

Carbon Dioxide Production

Production of carbon dioxide was not detected in the amended samples, whereas the amended samples with excess nitrate produced a small amount of CO_2. Carbon dioxide production in samples containing bentonite was significant (Figure 29, Tables 3(b)-4(b) Appendix D). Amended samples containing excess nitrate produced 1610 μmol of CO_2 at 200 days while the basic amended samples produced 786 moles. Carbon dioxide production was much higher in the glucose/bentonite samples than in the cellulose/bentonite samples, indicating the potential of the microorganisms to produce significant amounts of CO_2 under hypersaline conditions when a simple sugar is present. In addition, bentonite seems to enhance the overall gas production.

Nitrous Oxide Production

Accumulation of nitrous oxide was detected only in samples containing bentonite (Figure 30, Tables 3(c)-4(c) Appendix D). With excess nitrate, the N_2O concentration reached 585 μmol and then started to decline.

Summary

We examined glucose metabolism by the mixed inoculum used in the long-term experiments. Total gas production in samples incubated under aerobic conditions was not evident, but anaerobic samples produced significant amounts of gas, especially the samples with bentonite plus excess nitrate. Production of carbon dioxide in the aerobic samples was observed only in the amended and excess-nitrate samples without bentonite. Bentonite enhanced the activity of anaerobes. Carbon-dioxide production in anaerobic samples with excess nitrate reached a higher amount than any of the totals reached thus far in the long-term inundated experiment. Substantial amounts of N_2O also accumulated in the headspace of these samples.

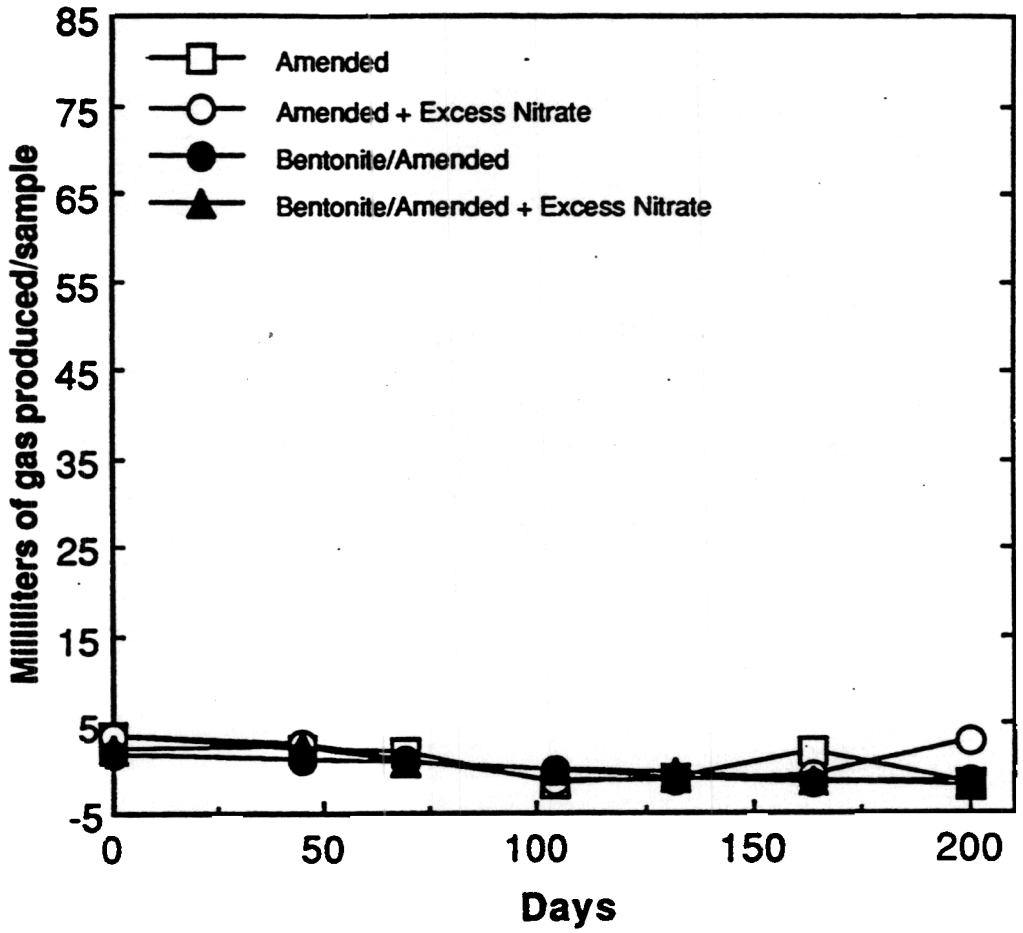

Figure 25. Total gas produced in samples containing glucose incubated with an initial atmosphere of air.

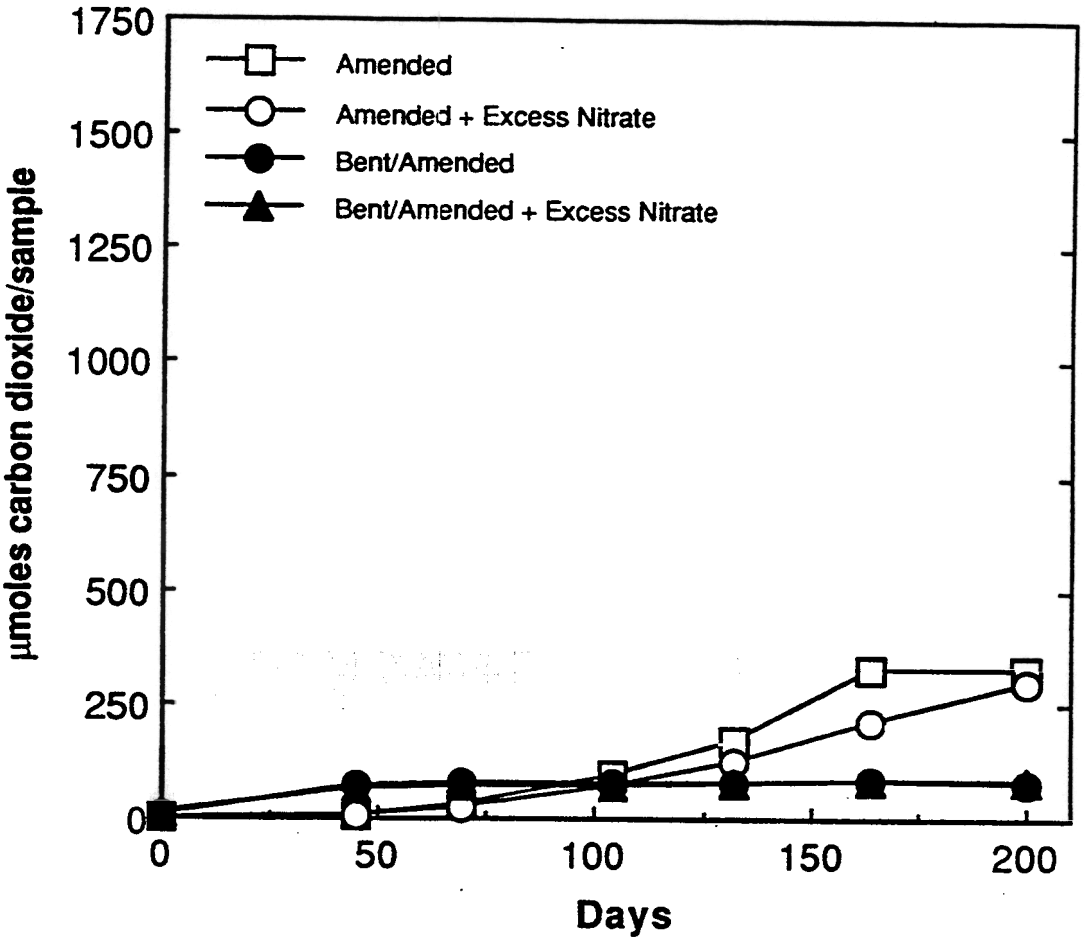

Figure 26. Carbon dioxide produced in samples containing glucose incubated with an initial atmosphere of air.

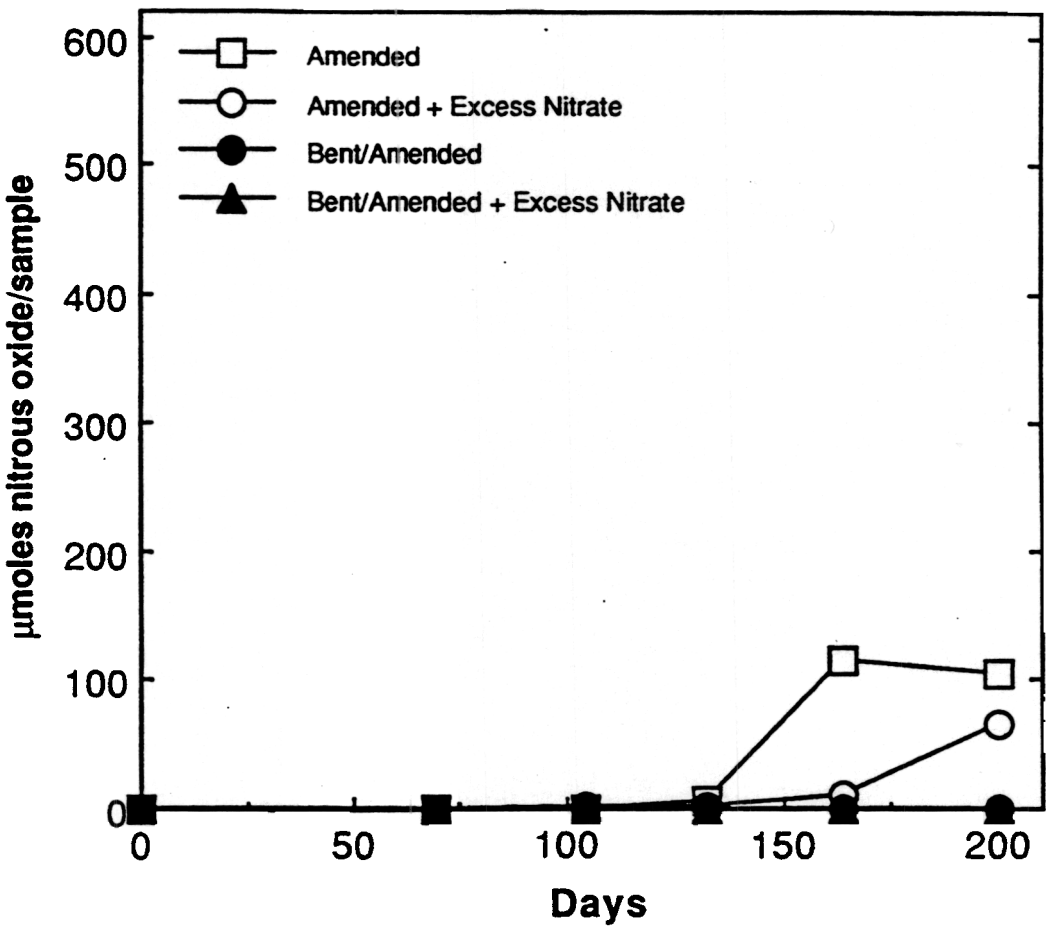

Figure 27. Nitrous oxide produced in samples containing glucose incubated with an initial atmosphere of air.

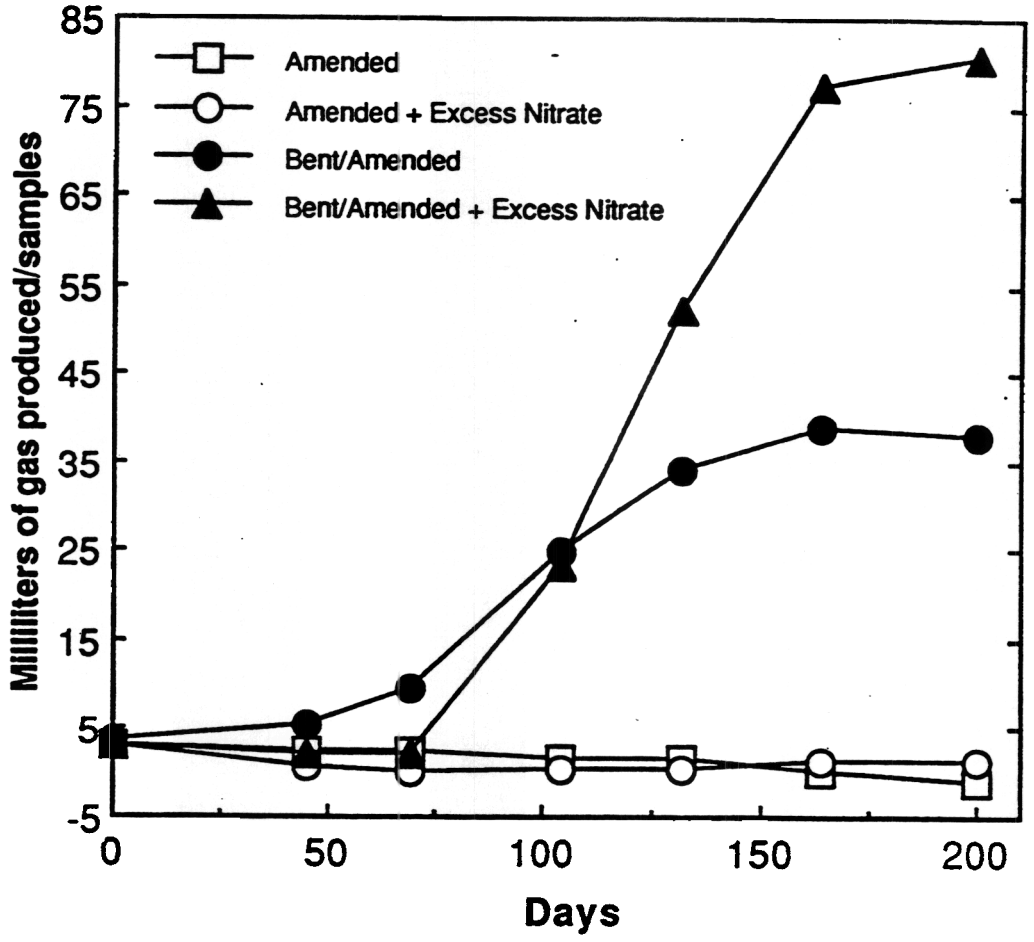

Figure 28. Total gas produced in anaerobic samples containing glucose.

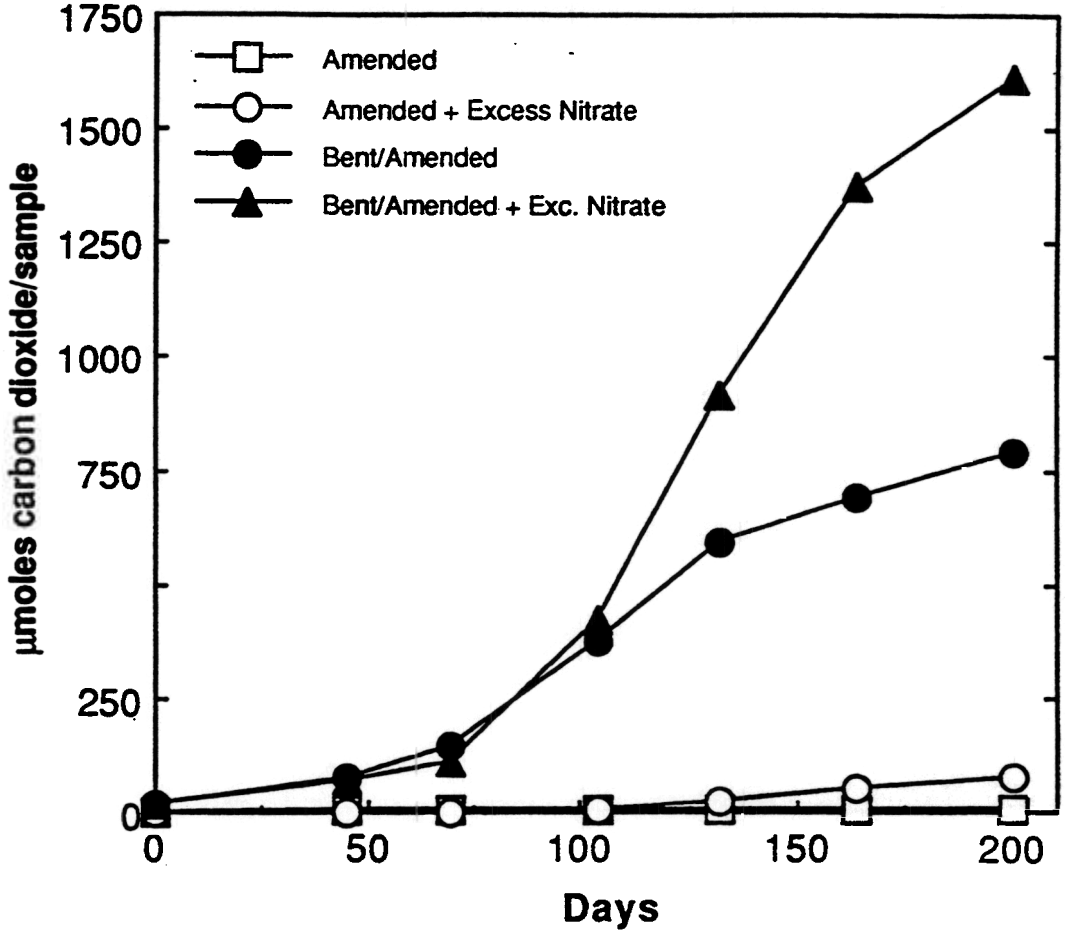

Figure 29. Carbon dioxide produced in anaerobic samples containing glucose.

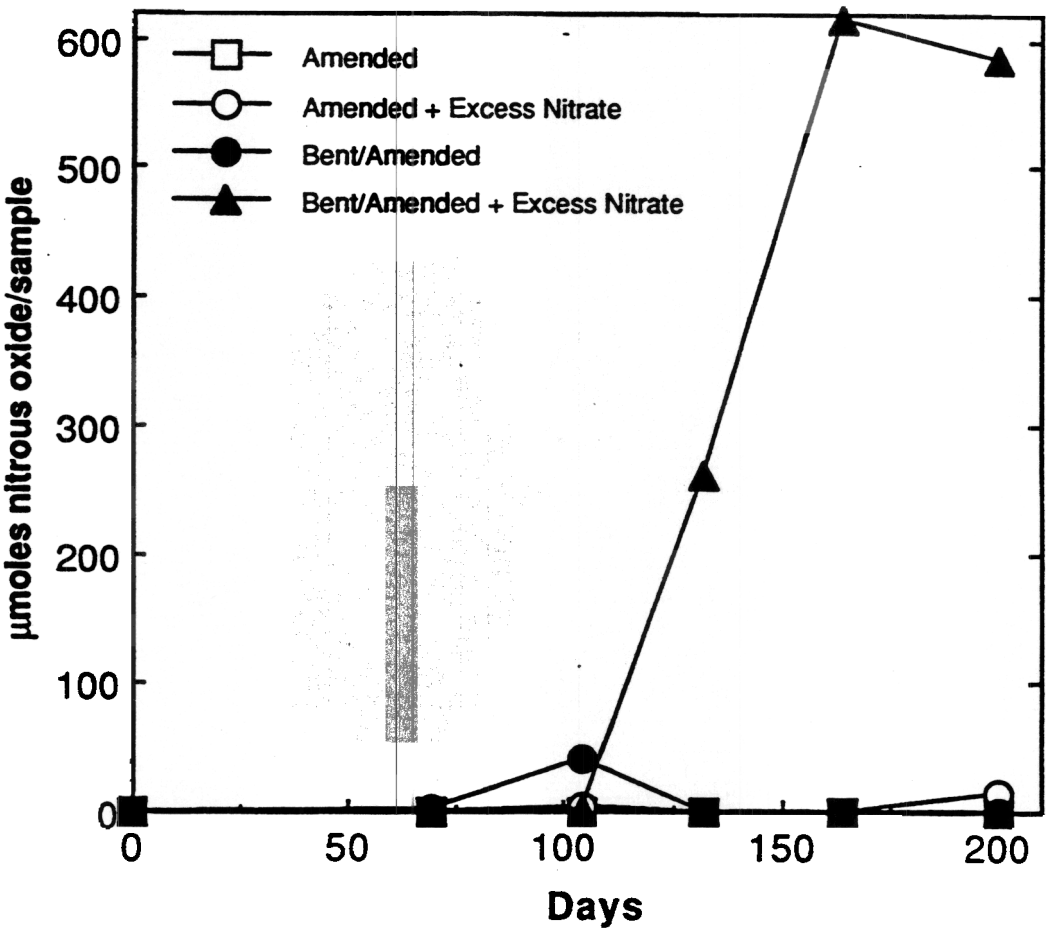

Figure 30. Nitrous oxide produced in anaerobic samples containing glucose.

DISTRIBUTION

Federal Agencies

US Department of Energy (6)
Office of Civilian Radioactive Waste Management
Attn: Deputy Director, RW-2
Associate Director, RW-10/50
Office of Program and Resources Management
Office of Contract Business Management
Director, RW-22
Analysis and Verification Division
Associate Director, RW-30
Office of Systems and Compliance
Associate Director, RW-40
Office of Storage and Transportation
Director, RW-4/5
Office of Strategic Planning and International Programs
Office of External Relations
Forrestal Building
Washington, DC 20585

US Department of Energy
Albuquerque Operations Office
Attn: National Atomic Museum Library
PO Box 5400
Albuquerque, NM 87185-5400

US Department of Energy (4)
WIPP Project Integration Office
Attn: W.J. Arthur III
L.W. Gage
P.J. Higgins
D.A. Olona
PO Box 5400
Albuquerque, NM 87115-5400

US Department of Energy (2)
WIPP Project Integration Satellite Office
Attn: R. Batra
R. Becker
PO Box 3090, Mail Stop 525
Carlsbad, NM 88221-3090

US Department of Energy
Research & Waste Management Division
Attn: Director
PO Box E
Oak Ridge, TN 37831

US Department of Energy (3)
WIPP Project Site Office (Carlsbad)
Attn: V. Daub
J. Lippis
J.A. Mewhinney
PO Box 3090
Carlsbad, NM 88221-3090

US Department of Energy
Attn: E. Young
Room E-178
GAO/RCED/GTN
Washington, DC 20545

US Department of Energy
Office of Environmental Restoration and Waste Management
Attn: J. Lytle, EM-30,
Trevion II
Washington, DC 20585-0002

US Department of Energy (3)
Office of Environmental Restoration and Waste Management
Attn: M. Frei, EM-34,
Trevion II
Washington, DC 20585-0002

US Department of Energy
Office of Environmental Restoration and Waste Management
Attn: S. Schneider, EM-342,
Trevion II
Washington, DC 20585-0002

US Department of Energy (2)
Office of Environment, Safety and Health
Attn: C. Borgstrom, EH-25
R. Pelletier, EH-231
Washington, DC 20585

US Department of Energy (2)
Idaho Operations Office
Fuel Processing and Waste
Management Division
785 DOE Place
Idaho Falls, ID 83402

US Environmental Protection
Agency (2)
Radiation Protection Programs
Attn: M. Oge
ANR-460
Washington, DC 20460

US Geological Survey (2)
Water Resources Division
Attn: R. Livingston
4501 Indian School NE
Suite 200
Albuquerque, NM 87110

US Nuclear Regulatory Commission
Division of Waste Management
Attn: H. Marson
Mail Stop 4-H-3
Washington, DC 20555

Boards

Defense Nuclear Facilities Safety Board
Attn: D. Winters
625 Indiana Ave. NW, Suite 700
Washington, DC 20004

Nuclear Waste Technical Review
Board (2)
Attn: Chairman
S.J.S. Parry
1100 Wilson Blvd., Suite 910
Arlington, VA 22209-2297

Advisory Committee on Nuclear Waste
Nuclear Regulatory Commission
Attn: R. Major
7920 Norfolk Ave.
Bethesda, MD 20814

State Agencies

Attorney General of New Mexico
P.O. Drawer 1508
Santa Fe, NM 87504-1508

Environmental Evaluation Group (3)
Attn: Library
7007 Wyoming NE
Suite F-2
Albuquerque, NM 87109

NM Energy, Minerals, and Natural
Resources Department
Attn: Library
2040 S. Pacheco
Santa Fe, NM 87505

NM Environment Department (3)
Secretary of the Environment
Attn: J. Espinosa
1190 St. Francis Drive
Santa Fe, NM 87503-0968

NM Bureau of Mines and Mineral
Resources
Socorro, NM 87801

NM Environment Department
WIPP Project Site
Attn: P. McCasland
PO Box 3090
Carlsbad, NM 88221

Laboratories/Corporations

Battelle Pacific Northwest Laboratories
Attn: R.E. Westerman, MSIN P8-44
Battelle Blvd.
Richland, WA 99352

INTERA, Inc.
Attn: G.A. Freeze
1650 University NE, Suite 300
Albuquerque, NM 87102

INTERA, Inc.
Attn: J.F. Pickens
6850 Austin Center Blvd., Suite 300
Austin, TX 78731

INTERA, Inc.
Attn: W. Stensrud
PO Box 2123
Carlsbad, NM 88221

IT Corporation
Attn: R.F. McKinney
Regional Office
5301 Central NE, Suite 700
Albuquerque, NM 87108

Los Alamos National Laboratory
Attn: B. Erdal, INC-12
PO Box 1663
Los Alamos, NM 87544

RE/SPEC, Inc.
Attn: W. Coons
4775 Indian School NE, Suite 300
Albuquerque, NM 87110-3927

RE/SPEC, Inc.
Attn: J.L. Ratigan
PO Box 725
Rapid City, SD 57709

Southwest Research Institute (2)
Center for Nuclear Waste Regulatory Analysis
Attn: P.K. Nair
6220 Culebra Road
San Antonio, TX 78228-0510

SAIC
Attn: H.R. Pratt
10260 Campus Point Dr.
San Diego, CA 92121

SAIC (2)
Attn: M. Davis
J. Tollison
2109 Air Park Rd. SE
Albuquerque, NM 87106

Tech Reps Inc. (3)
Attn: J. Chapman
C. Crawford
T. Peterson
5000 Marble NE, Suite 222
Albuquerque, NM 87110

TRW Environmental Safety Systems
Attn: L. Wildman
2650 Park Tower Dr., Suite 1300
Vienna, VA 22180-7306

Westinghouse Electric Corporation (5)
Attn: Library
C. Cox
L. Fitch
B.A. Howard
R. Kehrman
PO Box 2078
Carlsbad, NM 88221

Westinghouse—Savannah River Technology Center (4)
Attn: N. Bibler
J.R. Harbour
M.J. Plodinec
G.G. Wicks
Aiken, SC 29802

National Academy of Sciences, WIPP Panel

Howard Adler
Oak Ridge Associated Universities
Medical Sciences Division
PO Box 117
Oak Ridge, TN 37831-0117

Ina Alterman
Board on Radioactive Waste Management, GF456
2101 Constitution Ave.
Washington, DC 20418

Fred M. Ernsberger
1325 NW Tenth Ave.
Gainsville, FL 32605

John D. Bredehoeft
Western Region Hydrologist
Water Resources Division
US Geological Survey (M/S 439)
345 Middlefield Road
Menlo Park, CA 94025

Rodney C. Ewing
Department of Geology
University of New Mexico
Albuquerque, NM 87131

Charles Fairhurst
Department of Civil and Mineral Engineering
University of Minnesota
500 Pillsbury Dr. SE
Minneapolis, MN 55455-0220

B. John Garrick
PLG Incorporated
4590 MacArthur Blvd., Suite 400
Newport Beach, CA 92660-2027

Leonard F. Konikow
US Geological Survey
431 National Center
Reston, VA 22092

Carl A. Anderson, Director
Board on Radioactive Waste Management
National Research Council
HA 456
2101 Constitution Ave. NW
Washington, DC 20418

Jeremiah O'Driscoll
Jody Incorporated
505 Valley Hill Drive
Atlanta, GA 30350

Christopher G. Whipple
ICF Kaiser Engineers
1800 Harrison St., 7th Floor
Oakland, CA 94612-3430

Individuals

P. Drez
8816 Cherry Hills Rd. NE
Albuquerque, NM 87111

K. Lickliter
400-C 8th St. SW
Tacoma, WA 98439

D.W. Powers
Star Route Box 87
Anthony, TX 79821

Universities

University of Missouri-Rolla
Department of Nuclear Engineering
Attn: N. Tsoulfanidis
102 Fulton Hall
Rolla, MO 65401-0249

University of New Mexico
Geology Department
Attn: Library
141 Northrop Hall
Albuquerque, NM 87131

University of Washington
College of Ocean & Fishery Sciences
Attn: G.R. Heath
583 Henderson Hall HN-15
Seattle, WA 98195

Libraries

Thomas Brannigan Library
Attn: D. Dresp
106 W. Hadley St.
Las Cruces, NM 88001

Government Publications Department
Zimmerman Library
University of New Mexico
Albuquerque, NM 87131

New Mexico Junior College
Pannell Library
Attn: R. Hill
Lovington Highway
Hobbs, NM 88240

New Mexico State Library
Attn: N. McCallan
325 Don Gaspar
Santa Fe, NM 87503

New Mexico Tech
Martin Speere Memorial Library
Campus Street
Socorro, NM 87810

WIPP Public Reading Room
Carlsbad Public Library
101 S. Halagueno St.
Carlsbad, NM 88220

Foreign Addresses

Studiecentrum Voor Kernenergie
Centre d'Énergie Nucléaire
Attn: A. Bonne
SCK/CEN Boeretang 200
B-2400 Mol, BELGIUM

Atomic Energy of Canada, Ltd. (3)
Whiteshell Laboratories
Attn: B. Goodwin
M. Stevens
D. Wushke
Pinawa, Manitoba, CANADA R0E 1L0

Francois Chenevier (2)
ANDRA
Route du Panorama Robert Schumann
B.P. 38
92266 Fontenay-aux-Roses, Cedex
FRANCE

Jean-Pierre Olivier
OECD Nuclear Energy Agency
Division of Radiation Protection and Waste Management
38, Boulevard Suchet
75016 Paris, FRANCE

Claude Sombret
Centre d'Études Nucléaires de la Vallee Rhone
CEN/VALRHO
S.D.H.A. B.P. 171
30205 Bagnols-Sur-Ceze, FRANCE

Commissariat a L'Energie Atomique
Attn: D. Alexandre
Centre d'Études de Cadarache
13108 Saint Paul Lez Durance Cedex
FRANCE

Bundesanstalt für Geowissenschaften und Rohstoffe
Attn: M. Langer
Postfach 510 153
D-30631 Hannover, GERMANY

Bundesministerium für Forschung und Technologie
Postfach 200 706
5300 Bonn 2, GERMANY

Institut für Tieflagerung (2)
Attn: K. Kuhn
Theodor-Heuss-Strasse 4
D-3300 Braunschweig, GERMANY

Gesellschaft für Anlagen und Reaktorsicherheit (GRS) (2)
Attn: B. Baltes
W. Muller
Schwertnergasse 1
D-50667 Cologne, GERMANY

Physikalisch-Technische Bundesanstalt
Attn: P. Brenneke
Postfach 3345
D-3300 Braunschweig, GERMANY

Shingo Tashiro
Japan Atomic Energy Research Inst.
Tokai-Mura, Ibaraki-Ken, 319-11
JAPAN

Netherlands Energy Research Foundation ECN
Attn: L.H. Vons
3 Westerduinweg
PO Box 1
1755 ZG Petten
THE NETHERLANDS

Svensk Kärnbränsleforsorjning AB
Attn: F. Karlsson
Project KBS (Kärnbränslesakerhet)
Box 5864
S-102 48 Stockholm
SWEDEN